the nuclear donor cell into any enucleated recipient cell and transferring the recipient cell into any maternal host animal because the art of producing transgenic animals from any donor cell into any recipient cell and for transfer of artificial chromosomes in a cell that could serve as a donor cell was allegedly unpredictable.

To support this allegation, the Office Action states that the only teaching in the specification about nuclear transfer methods is on page 52, line 22 to page 53, line 7, which recites Wilmut et al. (1997) Nature 385:810-813, and "two world documents." (PCT applications). Of the cited references, the Examiner asserts that the two PCT applications, which are allegedly Applicant's own and have issued as U.S. Patents 6,025,155 and 6,077,697, do not provide any guidance as to how to produce transgenic animals by nuclear transfer. The Examiner further alleges that Wilmut *et al.* does not provide an enabling disclosure because this method has allegedly not been reproducible in other laboratories (citing Wolf *et al.*, J. Biotechnol., 65:99-110 (1998)).

The Examiner also provides numerous (post-effective filing date) references in support of other alleged "hurdles" that make the art of nuclear transfer "unpredictable", including: Stice *et al.* (*Theriogeneology* 49:129-138, (1998)), which allegedly teaches species-specific differences in animal cloning by nuclear transfer; Yanagimachi (*Mol. Cell. Endocrin.* 187:241-248 (2002)), which allegedly teaches that no single protocol of nuclear transfer works for all species because the characteristics of oocytes and donor cells are different from species to species; Oback and Wells (*Cloning and Stem Cells*, 4:169-174, (2002)), which allegedly demonstrates that donor cells selection and preparation was not routine even in 2002 and required extensive experimentation; Kuhholzer and Prather (*Soc. Exp. Biol. Med.*, 224:240-245, (2000)), which allegedly teaches that there is a "far from....perfect protocol" for nuclear transfer. The Examiner cites Co *et al.* (*Chromosome Res.*, 8:183-191, (2000)) as a reference that concededly shows that SATACS introduced into mouse embryos by

pronuclear microinjection are stable, but then goes on to assert that although this article teaches that the SATACS are stable, the introduction of a SATAC containing a heterologous sequence in a donor cell and use of the donor cell in producing a transgenic mammal "will be unpredictable" in view of the discussion above.

The Examiner concludes that it would require undue experimentation by one of skill in the art to practice the claimed methods because of the "unpredictable" state of the art of producing transgenic animals by nuclear transfer and because the specification allegedly does not teach how to address the limitations and "unpredictable" nature of the claimed subject matter.

This rejection is respectfully traversed.

First, it is noted that the instant application is a continuation of copending U.S. application Serial No. 08/835,682, filed April 10, 1997, is also a continuation-in-part of U.S. application Serial No. 08/695,191, filed August 7, 1996, now U.S. Patent No. 6,025,155, is also continuation-in-part of U.S. application Serial No. 08/682,080, filed July 15, 1996, now U.S. Patent No. 6,077,697, and is also a continuation-in-part of copending U.S. application Serial No. 08/629,822, filed April 10, 1996. The presently pending claims have an effective filing date of April 10, 1997.

Reliance upon post-filing date references to establish a lack of enablement is improper. Further, reliance upon references such as Co *et al.* in speculation that if, instead of the disclosed methods of pronuclear microinjection therein, they carry out a method of nuclear transfer, the use of the donor cell to produce a transgenic animal "will be unpredictable", is inapt since the technology therein does not establish any lack of enablement of the methods of nuclear transfer as instantly claimed. In fact, as discussed below, Co *et al.* teaches that satellite DNA-based artificial chromosomes (SATACS) might be especially well-suited for stable transfer and maintenance in cells, with few inter-species differences.

Furthermore, contrary to the Examiner's statement that the "two world documents" described and incorporated by reference into the specification at page 52, line 22 to page 53, line 7 are "Applicants'...own," the inventors of the two publications, International PCT application Nos. WO 97/07669 and WO 97/07668 (hereinafter "International Applications") of record in the instant application in an Information Disclosure Statement filed September 5, 2001), are two of the co-authors (Ian Wilmut and Keith Campbell) of Wilmut *et al.*, which is the third reference described and incorporated by reference therein. The two International Applications are owned, not by Applicant, but by the Roslin Institute (Edinburgh). Like Wilmut *et al.*, both International Applications provide methods of nuclear transfer that are described and incorporated by reference in the instant specification. Therefore, without conceding the propriety of the Examiner's statement that U.S. Patents 6,025,155 and 6,077,697, do not provide any guidance as to how to produce transgenic animals by nuclear transfer, it is respectfully submitted that the two International Applications described and incorporated by reference in the specification do provide guidance and protocols for carrying out nuclear transfer.

Relevant law

To satisfy the enablement requirement of 35 U.S.C § 112, first paragraph, the specification must teach one of skill in the art to make and use the invention without undue experimentation. Atlas Powder Co. v. E.I. DuPont de Nemours, 750 F.2d 1569, 224 USPQ 409 (1984). This requirement can be met by providing sufficient disclosure, either through illustrative examples or terminology, to teach one of skill in the art how to make and how to use the claimed subject matter without undue experimentation. This clause does not require "a specific example of everything *within the scope* of a broad claim." In re Anderson, 176 USPQ 331, at 333 (CCPA 1973), emphasis in original. Rather, the requirements of § 112, first paragraph "can be fulfilled by the use of

illustrative examples or by broad terminology." In re Marzocci et al., 469 USPQ 367 (CCPA 1971)(emphasis added).

Further, because "it is manifestly impracticable for an applicant who discloses a generic invention to give an example of every species falling within it, or even to name every such species, it is sufficient if the disclosure teaches those skilled in the art what the invention is and how to practice it." In re Grimme, Keil and Schmitz, 124 USPQ 449, 502 (CCPA 1960). Thus, there is no doubt that a patentee's invention may be broader than the particular embodiment shown in the specification. A patentee not only is entitled to narrow claims particularly directed to the preferred embodiment, but also to broad claims that define the invention without a reference to specific instrumentalities. Smith v. Snow, 294 U.S. 1, 11, 24 USPQ 26, 30 (1935).

Thus, there is no requirement for disclosure of every species within a genus. Applicant is entitled to claims are commensurate in scope not only with what applicant has specifically exemplified, but commensurate in scope with that which one of skill in the art could obtain by virtue of that which the applicant has disclosed.

The inquiry with respect to scope of enablement under 35 U.S.C. §112, first paragraph, is whether it would require undue experimentation to make and use the claimed invention. A considerable amount of experimentation is permissible, particularly if it is routine experimentation. The amount of experimentation that is permissible depends upon a number of factors, which include: the quantity of experimentation necessary, the amount of direction or guidance presented, the presence or absence of working examples, the nature of the invention, the state of the prior art, the relative skill of those in the art, the predictability of the art, and the breadth of the claims. Ex parte Forman, 230 USPQ 546 (Bd. Pat. App. & Int'f 1986); see also In re Wands, 8 USPQ2d 1400 (Fed. Cir. 1988).

Analysis

1. **Application of the Above-noted Factors**

Scope of the claims

Claims 23-60 are directed to methods including the introduction of an artificial chromosome into a nuclear donor cell and transferring the nucleus of the nuclear donor cell into an enucleated recipient cell. Dependent claims specify that the method further comprises transferring the recipient cell into a maternal host animal, permitting the transferred recipient cell to develop into a host, obtaining a nuclear donor cell from the fetus wherein the cell comprises an artificial chromosome, and transferring a nucleus from the fetal nuclear donor cell into a second enucleated recipient cell. Other dependent claims specify the type of host, the type of artificial chromosome including minichromosomes and satellite artificial chromosomes, the method by which the artificial chromosome is produced, the types of recipient cells, methods of introduction of the artificial chromosome into nuclear donor cells, culturing the recipient cells after transfer of the nuclear donor cell, and whether the artificial chromosome contains heterologous DNA encoding a gene product.

The steps of the methods claimed herein, including the preparation, characterization and isolation of artificial chromosomes, including SATACS such as satellite artificial chromosomes and minichromosomes, are described in the specification in extensive detail, and numerous examples of particular embodiments thereof are provided. The specification further describes methods of introducing artificial chromosomes into cells, incorporating heterologous DNA into artificial chromosomes, including minichromosomes and SATACs, expression of the heterologous DNA in cells containing artificial chromosomes and the use of such artificial chromosomes in gene therapy and in the preparation of transgenic animals. The specification provides numerous methods for introducing artificial chromosomes into cells for generating transgenic animals, including nuclear transfer methods such as fusion and microinjection.

Applicant is entitled to claims that are commensurate in scope not only with what applicant has specifically exemplified, but commensurate in scope with that which one of skill in the art could obtain by virtue of that which the applicant has disclosed. In the above-captioned application, Applicant discloses to the public methods and compositions for the controlled introduction and stable extra-genomic maintenance of large heterologous DNA fragments in cells without disruption of the inherent genome and, likewise, without the otherwise uncontrollable influences that the genomic DNA may have on the expression of the heterologous DNA. The artificial chromosomes disclosed in the application can be manipulated and used to express heterologous genes in cells, as is taught and specifically exemplified in the specification. It is clear that Applicant's discovery is of a pioneering nature, and, as such, is entitled to broad claim protection.

As taught in the above-captioned application, any methods known in the art pertaining to introduction of foreign genes carried in traditional, standard sources (such as genes harbored in expression vectors) into cells for any variety of purposes, e.g.,, gene therapy, protein production and the generation of transgenic animals, including nuclear transfer methods, may be applied in similar fashion to the introduction of artificial chromosomes, particularly SATACs and minichromosomes, into cells. The application describes and demonstrates that once the artificial chromosomes are generated and isolated and/or introduced into cells, then any known procedure that has previously been carried out with any heterologous gene from any source is applicable to utilization of artificial chromosomes carrying foreign genes of interest. The application is replete with descriptions of numerous uses of SATACs and minichromosomes. The descriptions of the many ways in which the artificial chromosomes may be used include references to reported procedures for introducing exogenous nucleic acids into cells.

It is therefore respectfully submitted that the claims directed to methods of nuclear transfer and, further, producing transgenic animals using SATACs and minichromosomes, are commensurate in scope with the discovery and its disclosure within the above-captioned application. It would be unfair and contrary to the Constitutional mandate set forth in Article, Section 8, to deprive Applicant of protection of the broad applications of the pioneering discovery disclosed and described in exhaustive detail in the subject application.

Level of Skill

The level of skill in this art is recognized to be high (see, *e.g.*, Ex parte Forman, 230 USPQ 546 (Bd. Pat. App. & Int'f 1986)). The numerous articles and patents made of record in this application address a highly skilled audience and further evidence the high level of skill in this art.

Teachings of the specification

The specification describes the production, characterization and isolation of artificial chromosomes and their transfection into cells or host organisms fo further nuclear transfer into a recipient cell and development into a transgenic animal. The teachings of the specification describe how to: (i) prepare artificial chromosomes; (ii) incorporate heterologous DNA encoding a product into an artificial chromosome; (iii) transfer the artificial chromosome containing the heterologous DNA into a cell, including a nuclear donor cell; (iv) transfer the nucleus of the nuclear donor cell into an enucleated recipient cell (v) transfer the recipient cell containing the artificial chromosome into a host animal; and (vi) permitting the recipient cell to develop into a fetus or animal in the host.

Each of these steps are described in detail in the specification. In addition, the specification provides numerous working examples of the procedures and results involved in the claimed methods. For example, the specification discloses methods for generating artificial chromosomes such as satellite artificial chromosomes, minichromosomes, and λ neochromosomes; methods for isolation and large-scale production of artificial chromosomes;

methods for delivery of artificial chromosomes to selected cells, including, for example, nuclear donor cells, the cells of a host animal, plant, or insect; methods for the introduction of cells comprising artificial chromosomes into a host animal, plant, or insect; methods for the expression of therapeutic products encoded by the nucleic acids of the artificial chromosomes in cell. Further, the specification discloses cell lines and chromosomes produced by the methods described, which can be used as vehicles for the expression of heterologous nucleic acids in cells *in vitro* and *in vivo*.

The specification discloses methods of generating artificial chromosomes, such as satellite artificial chromosomes and minichromosomes, and characterizes in exquisite detail the artificial chromosomes generated by such methods. To illustrate the methods and products thereof, the specification describes the exact procedures used repeatedly to generate multiple specific cell lines containing mammalian artificial chromosomes (see Examples 2-7), and the Applicant provides to the public no less than six of the described cell lines which have been deposited at an authorized depository (*i.e.*, the European Collection of Animal Cell Culture) (see page 74, line 23, through page 75, line 7).

The mammalian artificial chromosomes contained within these cells were extensively characterized using methods including Southern hybridization, long-range mapping of restriction endonuclease sites, indirect immunofluorescence with anti-centromere antibodies, *in situ* hybridization, analysis of G-band patterns, and chromosome painting. Such extensive analysis provides definition of the artificial chromosomes at the level of the basic structural and functional elements that comprise these chromosomes, including repeated units of satellite and foreign DNA. Many of these features are depicted schematically in Figures 1-3 of the application.

The specification further teaches methods of inserting heterologous DNA into the artificial chromosomes and the expression of the heterologous DNA

contained therein in cells (see, *e.g.*, page 39, line 25, through page 41, line 3; page 61, line 28, through page 62, line 7; page 150, line 1, through page 165, line 12 and Example 12 beginning on page 140). Procedures for the isolation of artificial chromosomes (see, *e.g.*, page 41, line 4, through page 42, line 3; page 32, lines 13-24; page 80, lines 20-27; and Example 10, beginning on page 124) and for the transfer of the artificial chromosomes into cells (see, *e.g.*, page 10, lines 25-31; page 48, line 11, through page 51, line 26; page 52, line 11, through page 55, line 3; page 70, line 14, through page 72, line 27; and Example 13 beginning on page 165) are also described in detail in the specification.

At page 11, lines 14-18, the specification describes how to introduce artificial chromosomes into cells for various uses, by using microinjection, cell fusion, microcell fusion, electroporation, electrofusion, projectile bombardment, nuclear transfer, calcium phosphate precipitation, lipid-mediated transfer systems and other such methods.

Additionally, the specification provides multiple possible uses of the artificial chromosomes with reference to procedures involved in those applications where appropriate. For example, the specification teaches on page 5, line 15, through page 6, line 8, that artificial chromosomes with integrated heterologous DNA may be used in methods of gene therapy, in methods of production of gene products, particularly products that require expression of multigene biosynthetic pathways, and also are intended for delivery into the nuclei of germline cells, such as embryo-derived stem cells [ES cells] for production of transgenic animals.

At page 52, line 11 through page 53, the specification describes in detail and incorporates by reference (Wilmut et al. (1997) Nature 385:810-813; WO 97/07668; WO 97/07669) methods, including that of nuclear transfer, for the production of transgenic animals. Microcell fusion to selected primary cells, somatic cells and embryonic stem cells to generate transgenic animals are

provided *e.g.*, at Example 1.A.5 beginning at page 70 of the specification. The various methods for generating transgenic animals are further exemplified in Example 14 beginning at page 168.

Rebuttal of the Examiner's Allegation that the Disclosure of Wilmut et al. (1997) Nature 385:810-813 is Not Enabling

The Examiner alleges that the article of Wilmut *et al.* does not provide an enabling disclosure because this method has not been reproducible in other laboratories (Wolf *et al.*, *J. Biotech.*, 65:99-110, (1998), page 101, first full paragraph in the right column).

It is respectfully submitted that the relevant question with regard to enablement of the subject matter of the instant claims is whether the particular steps and materials of the claimed methods are described in the specification in such a way as to enable one skilled in the art to make and use the subject matter **as claimed**. The specification provides and incorporates by reference (Wilmut et al. (1997) Nature 385:810-813, International PCT application Nos. WO 97/07669 and WO 97/07668) the steps of the methods as instantly claimed in which the SATAC containing the genes of interest is introduced by any suitable method, into an appropriate donor cell, such as a mammary gland cell, that contains totipotent nuclei; the diploid nucleus of the cell, which is either in G0 or G1 phase, is then introduced, such as by cell fusion or microinjection, into an unactivated oöcyte, preferably enucleated cell, which is arrested in the metaphase of the second meiotic division. The specification then provides that enucleation may be effected by any suitable method, such as actual removal, or by treating with means, such as ultraviolet light, that functionally remove the nucleus. The oöcyte is then activated, preferably after a period of contact, about 6-20 hours for cattle, of the new nucleus with the cytoplasm, while maintaining correct ploidy, to produce a reconstituted embryo, which is then introduced into a host. Ploidy is maintained during activation, for example, by incubating the reconstituted cell in the presence of a microtubule

inhibitor, such as nocodazole, colchicine, cocemid, and taxol, whereby the DNA replicates once. By following the teachings of the specification as provided herein, one of skill in the art can (1) introduce a chromosome into any cell, including a nuclear donor cell; (2) transfer the nucleus of the nuclear donor cell into an enucleated recipient cell; and (3) further transfer the recipient cell into a host animal for development of an animal or fetus therefrom.

This is evidenced by the success of practicing the nuclear transfer steps of the method as demonstrated by Wilmut et al. (1997) Nature 385:810-813, International PCT application Nos. WO 97/07669 and WO 97/07668, incorporated by reference herein. Contrary to the Examiner's assertion that the disclosure in Wilmut *et al.* is not enabling, Wilmut *et al.* (and International PCT application Nos. WO 97/07669 and WO 97/07668) demonstrated that by following the steps of the method as provided in the specification, live lambs born after nuclear transfer from a mammary gland cell were produced. Applicant is not aware of any requirement under current U.S. patent law specifying that the method must be fully reproducible every time it is practiced. Rather, a patent application satisfies the requirements of 35 U.S.C. § 112, first paragraph, as long as it provides sufficient disclosure, either through illustrative examples or terminology, to teach those of skill how to make and use the claimed subject matter with reasonable, but not undue, experimentation.

Moreover, the reliance on Wolf *et al.*, *J. Biotech.*, 65:99-110, (1998), (page 101, first full paragraph in the right column) for the proposition that the disclosure of Wilmut *et al.* is not enabling, is misplaced. The passage from Wolf *et al.* cited by the Examiner as establishing that the method has not been reproducible in other laboratories, merely mentions that the result in Wilmut *et al.* of producing "Dolly", an animal cloned by nuclear transfer from an adult cell, had not been reproduced as of that date and had been questioned. Wolf *et al.* cites Sgamarella *et al.* (*Science*, 279(51):635-636, (1998); attached hereto) for this assertion. Wolf *et al.* provides no disclosure as to whether the steps of the

method, as actually practiced by one of skill in the art, would involve undue experimentation.

A review of Sgamarella *et al.* indicates that this reference is critical of the success of Wilmut *et al.*, but again provides no evidence demonstrating that the disclosure of Wilmut *et al.* is not enabling. Sgamarella *et al.* is skeptical of the reproducibility of the method of Wilmut *et al.* only because Wilmut allegedly announced that he had no intention of practicing the method again. Sgamarella *et al.* further expresses skepticism because a subsequent publication of Wilmut used a fetal cell rather than an adult cell in a nuclear transfer method, leading Sgamarella *et al.* to question whether Wilmut *et al.* used an adult cell and, if so, why "Dolly's" mitochondrial DNA was not analyzed to establish the same.

Neither an alleged decision by Wilmut not to practice the method of Wilmut *et al.* again, nor any of the allegations in Sgamarella *et al.* provide any evidence that the disclosure in Wilmut *et al.* is not enabling. Moreover, in a paragraph (attached hereto) immediately following the article by Sgamarella *et al.*, Wilmut provided explanations for each of the allegations in Sgamarella *et al.*, none of which go to lack of enablement of the disclosure in Wilmut *et al.*

As further evidence that the disclosure in Wilmut *et al.* is enabling, attached hereto is U.S. Patent No. 6,147,276, filed February 19, 1997, based on the disclosure in Wilmut *et al.* The patent, which claims the method of nuclear transfer as set forth in Wilmut *et al.*, is presumptively valid (35 U.S.C. 282) and therefore presumed enabled.

Notwithstanding the above arguments, to evidence that the methods as claimed operate as claimed, attached are publications (Schnieke *et al.*, *Science*, 278:2130-2133, (1997); WO 02/062131) demonstrating that by following the teachings of the application and that of Wilmut *et al.* incorporated therein, nuclear transfer and the generation of transgenic animals is obtained as claimed.

Also, although these publications rebut assertions of inoperativeness, they also further evidence enablement. It is noted that the level of skill in the

biotechnical arts is recognized to be high (see, *e.g.*, *Ex parte Forman*, 230 USPQ 546 (Bd. Pat. App. & Int'f 1986). Further, as discussed below, methods for performing the various steps of the claimed methods, such as introducing nucleic acids including chromosomes into nuclear donor cells, transferring the nuclei of the nuclear donor cells into recipient cells and allowing the recipient cell to develop into a transgenic fetus or animal in a host are known to the skilled artisan.

The reference Schnieke *et al.* demonstrates that by following the steps of nuclear transfer of Wilmut *et al.*, live transgenic sheep encoding human Factor IX were produced (*e.g.*, Table 1 at page 2131). The International Publication WO 02/062131 demonstrates that by following the steps of the method as taught in the instant specification (1) artificial chromosomes such as SATACS can be generated and introduced into nuclear donor cells; and (2) the nucleus of the nuclear donor cell can be transferred to into an enucleated recipient cell to yield bovine blastocysts. These blastocysts can then be placed in extended embryo culture and are capable of generating animals (bovines) (Examples 1-3, *e.g.*, beginning at page 55 of the specification).

In summary, the specification enables one of skill in the art to, by following the methods set forth therein, generate artificial chromosomes, readily identify the resulting artificial chromosomes based on the detailed characterization provided in the specification, incorporate foreign nucleic acid, e.g., heterologous DNA encoding a therapeutic product, into an artificial chromosome, and isolate and transfer artificial chromosomes for use in other cells and systems, including the generation of transgenic animals by nuclear transfer and other such methods. By virtue of Applicant's discovery of artificial chromosomes and the teachings of the specification, those of ordinary skill in the art are able, without undue experimentation, to make and use the artificial chromosomes and to combine the artificial chromosomes with known recombinant DNA procedures, many of which are referenced in the

specification, to achieve any number of particular outcomes, including the introduction and stable maintenance of artificial chromosomes in cells, such as nuclear donor cells and obtaining transgenic animals by nuclear transfer and other such methods.

In summary, by following the methods set forth in the specification, the specification teaches one of skill in the art how to generate artificial chromosomes, readily identify the resulting artificial chromosomes based on the detailed characterization provided in the specification, incorporate foreign nucleic acid, *e.g.*, heterologous DNA encoding a gene product into an artificial chromosome, isolate and transfer artificial chromosomes in to nuclear donor cells for use in nuclear transfer methods, and practice nuclear transfer methods.

Knowledge of those of skill in the art

At the time of filing of the application, a broad body of knowledge had amassed in the area of the manipulation of DNA and recombinant DNA techniques for use in gene therapy and transgenic animal applications. Numerous such procedures are referenced in the instant application, for example, as follows.

Procedures relating to DNA manipulation and the insertion of heterologous DNA, including transgenes encoding selective markers and therapeutic products, into an artificial chromosome are found throughout the application. For example, Example 12, beginning at page 140 in the specification, describes the preparation of chromosome fragmentation vectors and other vectors for targeted integration of heterologous DNA into MACs. These plasmids and other vectors were prepared using the numerous general procedures for the manipulation of DNA, including the preparation of DNA probes and plasmids referenced throughout the application as seen, for example, at page 73, line 23, through page 74, line 21, (see, *e.g.*, Sambrook *et al.* (1989) *Molecular cloning: A Laboratory Manual Cold Spring Harbor Laboratory Press,* Cold Spring Harbor, NY, Wong *et al.* (1988) *Nucl. Acids Res. 16*:11645-11661, Fatyol *et al.* (1994)

Nucl. Acids Res. 22:3728-3736, Morgenstern *et al.* (1990) *Nucl. Acids Res. 18*:3587-3596, Tonghua *et al.* (1995) *Chin. Med. J. (Beijing, Engl. Ed.) 108*:653-659, Couto *et al.* (1994) *Infect. Immun. 62*:2375-2378, Dunckley *et al.* (1992) *FEBS Lett. 296*:128-34, French *et al.* (1995) *Anal. Biochem. 228*:354-355, Liu *et al.* (1995) *Blood 85*:1095-1103, International PCT application Nos. WO 95/20044, WO 95/00178, and WO 94/19456).

Procedures for the introduction of heterologous nucleic acids, including targeting vectors and artificial chromosomes, into cells and host animals are referred to in many instances throughout the application. For example, at page 23, lines 18-31, to page 24, lines 1-11, the application references numerous procedures for the introduction of DNA or RNA into cells, including for example direct uptake using calcium phosphate (*e.g.*, Wigler *et al.* (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*:1373-1376), electroporation, lipofection, polyethylene glycol-mediated DNA uptake (*e.g.*, Strauss (1996) *Meth. Mol. Biol. 54*:307-327), microcell fusion (*e.g.*, Lambert (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*:5907-5911; U.S. Patent No. 5,396,767, Sawford *et al.* (1987) *Somatic Cell Mol. Genet. 13*:279-284; Dhar *et al.* (1984) *Somatic Cell Mol. Genet. 10*:547-559; and McNeill-Killary *et al.* (1995) *Meth. Enzymol. 254*:133-152), and lipid-mediated carrier systems (*e.g.*, Teifel *et al.* (1995) *Biotechniques 19*:79-80; Albrecht *et al.* (1996) *Ann. Hematol. 72*:73-79; Holmen *et al.* (1995) *In Vitro Cell Dev. Biol. Anim. 31*:347-351; Remy *et al.* (1994) *Bio Conjug. Chem. 53*:647-654; Le Bolch *et al.* (1995) *Tetrahedron Lett. 36*:6681-6684; Loeffler *et al.* (1993) *Meth. Enzymol. 217*:599-618);

At page 48, lines 16 through page 51, line 26, the application refers to several methods of introducing nucleic acids, including targeting vectors and artificial chromosomes, into different types of cells, such as cell or microcell fusion, direct DNA transfer, electroporation, lipid-mediated transfer, *e.g.*, lipofection and liposomes, microprojectile bombardment, microinjection in cells and embryos, protoplast regeneration for plants, and any other suitable method

(see, *e.g.*, Weissbach *et al.* (1988) *Methods for Plant Molecular Biology*, Academic Press, N.Y., Section VIII, pp. 421-463; Grierson *et al.* (1988) *Plant Molecular Biology, 2d Ed.*, Blackie, London, Ch. 7-9; see, also U.S. Patent Nos. 5,491,075; 5,482,928; and 5,424,409; see, also, *e.g.*, U.S. Patent No. 5,470,708, which describes particle-mediated transformation of mammalian unattached cells).

Other methods for introducing nucleic acid into cells include nuclear microinjection and bacterial protoplast fusion with intact cells. Polycations, such as polybrene and polyornithine, can also be used. For various techniques for transforming mammalian cells, see *e.g.*, Keown *et al.* (1990) *Methods in Enzymology 185*:527-537; and Mansour *et al.* (1988) *Nature 336*:348-352. Isolated, purified artificial chromosomes can be injected into an embryonic cell line or embryonic stem cells (see, *e.g.*, Hogan *et al.* (1994) *Manipulating the Mouse Embryo, A :Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, see, especially, pages 255-264 and Appendix 3).

At page 49, line 15, to page 51, line 26, the specification refers to the practice of introducing nucleic acid into particular cell types by using standard techniques appropriate for such types of cells. For example, for mammalian cells that do not have cell walls, the calcium phosphate precipitation method for introduction of exogenous DNA (see, *e.g.*, Graham *et al.* (1978) *Virology 52*:456-457; Wigler *et al.* (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*:1373-1376; and *Current Protocols in Molecular Biology, Vol. 1,* Wiley Inter-Science, Supplement 14, Unit 9.1.1-9.1.9 (1990)) is often preferred. DNA uptake can be accomplished by DNA alone or in the presence of polyethylene glycol (PEG-mediated gene transfer), which is a fusion agent, or by any variations of such methods known to those of skill in the art (see, *e.g.*, U.S. Pat. No. 4,684,611).

Lipofection (see, *e.g.*, Strauss (1996) *Meth. Mol. Biol. 54*:307-327) can also be used to introduce DNA into cells. This method is particularly well-suited

for transfer of exogenous DNA into chicken cells (*e.g.*, chicken blastodermal cells and primary chicken fibroblasts; see Brazolot *et al.* (1991) *Mol. Repro. Dev. 30*:304-312). Additional methods useful in the direct transfer of DNA into cells include particle gun electrofusion (see, *e.g.*, U.S. Patent Nos. 4,955,378, 4,923,814, 4,476,004, 4,906,576 and 4,441,972) and virion-mediated gene transfer. The transfer of plasmid DNA in liposomes directly to human cells *in situ* has been approved by the FDA for use in humans (see, *e.g.*, Nabel, *et al.* (1990) *Science 249*:1285-1288 and U.S. Patent No. 5,461,032).

Chromosomes and nucleic acids can also be transferred by preparing microcells containing an artificial chromosome or nucleic acid and then fusing with selected target cells. Methods for such preparation and fusion of microcells are well known (see the Examples and also see, *e.g.*, U.S. Patent Nos. 5,240,840, 4,806,476, 5,298,429, 5,396,767, Fournier (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*:6349-6353; and Lambert *et al.* (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*:5907-59). Microcell fusion, using microcells that contain an artificial chromosome, is a particularly useful method for introduction of MACs into avian cells, such as DT40 chicken pre-B cells (for a description of DT40 cell fusion (see, *e.g.*, Dieken *et al.* (1996) *Nature Genet. 12*:174-182).

At page 52, lines 14-18, the specification references how transgenic (non-human) animals can be produced by introducing exogenous genetic material into a pronucleus of a mammalian zygote by microinjection (*see, e.g.*, U.S. Patent Nos. 4,873,191 and 5,354,674; see, also, International PCT application publication No. WO 95/14769, which is based on U.S. application Serial No. 08/159,084); and page 52, lines 22-23 references nuclear transfer methods for the generation of transgenic animals that were known to those of skill in the art (*see*, Wilmut *et al.* (1997) *Nature* 385:810-813, International PCT application Nos. WO 97/07669 and WO 97/07668). Nuclear transfer methods were also known to those of skill in the art prior to or at the earliest priority date of the intant methods or shortly therafter (*see, e.g.*, Wells *et al.*, *Biol. Reprod.*,

57:385-393 (1997), of record; Campbell *et al.* (1996) *Nature 380*:64-66; PCT Application Publication No. WO95/17500, attached hereto; Solter (1996) *Nature*, 380: 24-25, attached hereto; Sims *et al.* (1993), *Proc. Natl. Acad. Sci. USA*, 90:6143-6147, attached hereto; Tanaka *et al.* (1997) *Animal Reprod. Sci.*, 49:113-123, attached hereto).

These references to numerous published protocols for DNA manipulation, recombinant DNA expression, transfer of DNA (artificial chromosomes) into cells, including nuclear donor cells, and carrying out nuclear transfer methods for the generation of transgenic animals demonstrate the large volume of information regarding tested and reliable procedures available at the time of filing of the instant application and thus evidence the advanced state of the art at the relevant time.

Presence of Working Examples

The specification provides numerous working examples and descriptions of the construction, isolation and use of artificial chromosomes containing heterologous nucleic acid, the introduction of these artificial chromosomes into cells and the generation of transgenic animals from these cells containing artificial chromosomes or by direct introduction of the artificial chromosomes into cells.

Example 1.A.5 beginning at page 70 of the specification describes microcell fusion procedures that can be used in the generation of transgenic animals. Example 2, at page 75 of the specification, describes in great detail the preparation and maintenance of cell lines, including EC3/75, EC3/7C5 and EC3/7C6, which contain artificial chromosomes, as well as the assays used to monitor the expression of the *neo* gene encoded by the artificial chromosomes within the cells. Example 3, at page 81 of the specification, describes in great detail the transfer of the minichromosome (MMCneo) described in Example 2 into different mammalian cells, including hamster and human, through fusion of microcells, demonstrating the tolerance of a wide variety of cells for the artificial

chromosome. Example 13, at page 165 of the specification, describes methods for the microinjection of artificial chromosomes into eukaryotic cells, and detection of expression of the encoded heterologous DNA (βGal) in cells injected with the DNA. Example 14, at page 168 of the specification, describes in great detail the development of transgenic mice expressing the anti-HIV ribozyme encoded by an artificial megachromosome. Example 14 also describes in great detail the uses of the artificial chromosomes in generating transgenic animals.

Predictability

As is known to those of skill in the art (described above), the level of knowledge and skill in the preparation, isolation and use of artificial chromosomes, their introduction into cells and their further manipulation in methods such as nuclear transfer, microcell fusion or direct introduction into host organisms for the development of transgenic animals or fetuses as claimed in the instant application was high as of the effective filing date.

The pending claims are directed to methods in which an artificial chromosome is introduced into a nuclear donor cell, the nucleus of the nuclear donor cell is transferred into an enucleated recipient cell and the recipient cell may further be permitted to develop into an animal or a fetus in a host. The claims are directed to methods comprising specific steps that, in light of the teachings of the specification and the broad body of knowledge in the art pertaining to each of these steps, makes each of the steps of the method predictable.

The Examiner points to several references, including: Stice *et al.* (*Theriogeneology* 49:129-138, (1998)), which allegedly teaches species-specific differences in animal cloning by nuclear transfer; Yanagimachi (*Mol. Cell. Endocrin.* 187:241-248 (2002)), which allegedly teaches that no single protocol of nuclear transfer works for all species because the characteristics of oocytes and donor cells are different from species to species; Oback and Wells (*Cloning and Stem Cells*, 4:169-174, (2002)), which allegedly demonstrates that donor

cells selection and preparation was not routine even in 2002 and required extensive experimentation; Kuhholzer and Prather (*Soc. Exp. Biol. Med.*, 224:240-245, (2000)), which allegedly teaches that there is a "far from....perfect protocol" for nuclear transfer, for the proposition that the state of the art of nuclear transfer was and is "unpredictable". The Examiner also cites Co *et al.* (*Chromosome Res.*, 8:183-191, (2000)) as a reference that concededly shows that SATACS introduced into mouse embryos by pronuclear microinjection are stable, but then goes on to assert that although this article teaches that the SATACS are stable, the introduction of a SATAC containing a heterologous sequence in a donor cell and use of the donor cell in producing a transgenic mammal "will be unpredictable" in view of the above assessment of the state of the art.

First, it is respectfully submitted that reliance upon post-filing date references to establish a lack of enablement is improper. Further, reliance upon references such as Co *et al.* in speculation that if, instead of the disclosed methods of pronuclear microinjection therein, they carry out a method of nuclear transfer, the use of the donor cell to produce a transgenic animal "will be unpredictable", is inapt since the technology therein does not establish any lack of enablement of the methods of nuclear transfer as instantly claimed. To the contrary, Co *et al.* teaches that satellite DNA-based artificial chromosomes (SATACS) can be successfully delivered to (by pronuclear microinjection) and stably maintained and expressed in transgenic animals, with few inter-species differences (murine *vs.* bovine, *see, e.g.,* pp. 183-184), thus evidencing the desirability of using artificial chromosomes in methods for the generation of transgenic animals.

Further, it is respectfully submitted that a selective reading of the Stice *et al.* (*Theriogeneology* 49:129-138, (1998)); Yanagimachi (*Mol. Cell. Endocrin.* 187:241-248 (2002)); Oback and Wells (*Cloning and Stem Cells*, 4:169-174, (2002)); and Kuhholzer and Prather (*Soc. Exp. Biol. Med.*, 224:240-245,

(2000)) references, in which statements regarding the limitations of nuclear transfer are taken out of context, has resulted in a mischaracterization of the references that cannot validly be relied on to support an allegation of unpredictability of nuclear transfer.

For example, Stice *et al.* (*Theriogeneology* 49:129-138, (1998)), which allegedly teaches the limitations of species-specific differences in animal cloning by nuclear transfer that led to only one example of a nuclear transfer pig, in fact is a review on the "power and capabilities" of nuclear transfer, the breakthroughs in nuclear transfer, and the commercial applications of cloning by nuclear transfer. Stice *et al.* discusses how the commercial use of nuclear transfer is not limited by inefficiencies in nuclear transfer procedures, because only a few cloned transgenic founder animals are needed (*see, e.g.*, page 133, "Commercial Applications of Cloning").

Yanagimachi (*Mol. Cell. Endocrin.* 187:241-248 (2002)), which allegedly teaches that no single protocol of nuclear transfer works for all species because the characteristics of oocytes and donor cells are different from species to species, provides several examples of the successes in nuclear transfer (page 243, col. 2) and states that the relatively high cloning success rate in Japanese black cattle may provide clues as to how to solve low mortality issues in other species.

Oback and Wells (*Cloning and Stem Cells*, 4:169-174, (2002)), which allegedly demonstrates that donor cell selection and preparation was not routine even in 2002 and required extensive experimentation, discusses how standards in the cloning field are "currently poor," but then goes on to provide detailed guidelines for donor cell selection, cell cycle synchronization and other parameters for nuclear transfer (see entire publication). The availability of such detailed guidelines as provided in Oback and Wells establishes predictability rather than unpredictability of the practice of nuclear transfer methods.

Kuhholzer and Prather (*Soc. Exp. Biol. Med.*, 224:240-245, (2000)), while acknowledging that the limitations in practicing nuclear transfer, also discusses the promise of nuclear transfer as evidenced by the dramatic improvement in nuclear transfer protocols within the five preceding years, and how cloning and transgenic animal production have been greatly enhanced by the development of nuclear transfer technology (*see, e.g*, Abstract and page 244, col. 1).

Applicant is not aware of any requirement under current U.S. patent law specifying particular minimum levels of optimization and certified efficacy in order for a treatment-related area of art to qualify as sufficiently "predictable" such that lack of enablement under 35 U.S.C. § 112, first paragraph, is not a consideration. The relevant standard is not that of an established, fully optimized, clinical course of treatment; rather, even in an *unpredictable* art, a patent application satisfies the requirements of 35 U.S.C. § 112, first paragraph, as long as it provides sufficient disclosure, either through illustrative examples or terminology, to teach those of ordinary skill how to make and use the claimed subject matter with reasonable, but not undue, experimentation. There is no requirement that a treatment method achieve a specified level of efficacy or efficiency in order to be considered "enabled" by the specification.

Contrary to the position set forth in the Office Action, the cited references show that nuclear transfer is feasible and commercially promising, with in fact very low efficiency of nuclear transfer sufficing to establish a transgenic animal line.

It appears that the Examiner, in asserting the unpredictability of the art of nuclear transfer, has equated "limitations" with "unpredictability." It is respectfully submitted that although methods of nuclear transfer may be associated with certain limitations and limited success, this does not establish the art as unpredictable. In fact, with respect to methods of nuclear transfer, the well-studied, -identified and -characterized limitations of the art (*e.g.*, as provided in Oback and Wells), as determined through years of research, make

the methods all the more predictable. The practitioner is well aware of the potential obstacles and clearly knows what he or she is up against in designing and carrying out such methods. As such, it is respectfully submitted, that although the art of nuclear transfer may not have been a routine, clinical practice at the effective filing date of the subject application, it was not so unpredictable as to qualify as a major factor in the determination of whether the requirements of 35 U.S.C. § 112, first paragraph, are satisfied with respect to the instantly claimed subject matter.

Conclusions

In light of the extensive teachings and examples in the specification, the high level of skill of those in this art, the knowledge of those of skill in the art, the fact that it is predictable that artificial chromosomes can be introduced into cells and can be further used to generate transgenic animals by various methods such as nuclear transfer, and the breadth of the claims, it would not require undue experimentation for one of skill in the art to practice the claimed methods.

Accordingly, a consideration of the factors enumerated in Ex parte Forman leads to the conclusion that undue experimentation would not be required to introduce an artificial chromosome into a nuclear donor cell, transfer the nucleus of the nuclear donor cell into an enucleated recipient cell and further permit the recipient cell to develop into an animal or fetus in a host.

* * *

In view of the above amendments and remarks, reconsideration and allowance of the application are respectfully requested.

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(54) Title: CLONING OF TRANSGENIC ANIMALS COMPRISING ARTIFICIAL CHROMOSOMES

(57) Abstract: The invention is directed in part to totipotent cells that have one or more artificial chromosomes; processes for producing such cells; processes for using such cells (e.g., nuclear transfer); transgenic embryos and transgenic animals cloned from such cells; and processes for producing such embryos and animals.

CLONING OF TRANSGENIC ANIMALS COMPRISING ARTIFICIAL CHROMOSOMES

INTRODUCTION

The invention relates in part to the cloning of animals that comprise heterologous DNA molecules. Such transgenic animals preferably contain at least about 100 kilobase pairs of exogenous DNA in structures known to the skilled artisan as "artificial chromosomes."

BACKGROUND OF THE INVENTION

The following discussion of the background of the invention is merely provided to aid the reader in understanding the invention and is not admitted to describe or constitute prior art to the present invention.

Researchers have been developing methods for cloning mammalian animals over the past two decades. These reported methods typically include the steps of (1) isolating a pluripotent or totipotent cell; (2) inserting the cell or nucleus isolated from the cell into an enucleated oocyte (*i.e.*, the oocyte's nucleus was previously extracted), and (3) allowing the embryo to mature *in vivo*.

The first successful nuclear transfer experiment using mammalian cells was reported in 1983, when pronuclei isolated from a murine (mouse) zygote were inserted into an enucleated oocyte and resulted in like offspring(s). *See, e.g.*, McGrath & Solter, 1983, *Science 220*:1300-1302. Subsequently, other workers described the production of chimeric murine embryos (*e.g.*, embryos that contain a subset of cells having significantly different nuclear DNA from other cells in the embryo) using murine primordial germ cells (PGCs). These cells are and can give rise to pluripotent cells (*e.g.*, cells that can differentiate into other types of cells, and which may, but are not required to, differentiate into a grown animal). *See, e.g.*, Matsui *et al.*, 1992, *Cell* 70:841-847 and Resnick *et al.*, 1992, *Nature 359*:550; Kato *et al.*, 1994, *Journal of Reproduction and Fertility* Abstract Series, Society For the Study of Fertility, Annual Conference, Southampton, 13:38.

Progress has also been reported in the field of cloning ovine (sheep) animals (*see, e.g.*, Willadsen, 1986, *Nature 320*:63-65; Campbell *et al.*, 1996, *Nature 380*:64-66; PCT Publication WO 95/20042; Wilmut *et al.*, 1997, *Nature 385*:810-813; PCT Publication WO 96/07732; PCT Publication WO 97/07668; and PCT publication WO 97/07669; and McCreath *et al.*, 2000, *Nature*, 405:1066-1069), and bovine animals, (*see, e.g.*, U.S. Patents 4,994,384 and 5,057,420; Sims & First, 1993, *Theriogenology 39*:313; Keefer *et al.*, 1994, *Mol. Reprod. Dev. 38*:264-268; Delhaise *et al.*, 1995, *Reprod. Fert. Develop. 7*:1217-1219; Lavoir 1994, *J. Reprod. Dev.* 37:413-424; Stice *et al.*, 1996, *Biol. Reprod. 54*: 100-110; and PCT application WO 95/10599 entitled "Embryonic Stem Cell-Like Cells").

Researchers have also disclosed methods that resulted in cloned bovine animals (cattle). Bovines have been cloned using an embryonic cell derived from a 2-64 cell embryo as a nuclear donor. This bovine animal was reportedly cloned by utilizing nuclear transfer techniques set forth in U.S. Patents 4,994,384 and 5,057,420. Others reported that cloned bovine embryos were formed where an inner cell mass cell of a blastocyst stage embryo was utilized as a nuclear donor in a nuclear transfer procedure (Sims & First, 1993, *Theriogenology 39*:313; Keefer *et al.*, 1994, *Mol. Reprod. Dev. 38*:264-268; and U.S. Patent No. 6,107,543); a PGC isolated from fetal tissue as a nuclear donor (Delhaise *et al.*, 1995, *Reprod. Fert. Develop. 7*:1217-1219; Lavoir 1994, *J. Reprod. Dev. 37*:413-424; and PCT application WO 95/10599 entitled "Embryonic Stem Cell-Like Cells"); a proliferating somatic cell (U.S. Patent No. 5,945,577); and a reprogrammed nonembryonic cell (U.S. Patent No. 6,011,197)

Additionally, researchers have reported methods for obtaining cloned porcine animals and porcine chimeric animals, specifically, where a nuclear donor obtained from a 4-cell embryo is placed inside an enucleated zygote. *See, e.g.*, Prather *et al.*, 1989, *Biology of Reproduction 41*: 414-418; Piedrahita *et al.*, 1998, *Biology of Reproduction 58*: 1321-1329; and WO 94/26884, "Embryonic Stem Cells for Making Chimeric and Transgenic Ungulates," Wheeler, published November 24, 1994. Also, researchers have reported nuclear transfer experiments using porcine nuclear donors and porcine oocytes. *See., e.g.*, Nagashima *et al.*, 1997, *Mol. Reprod. Dev. 48*:.339-343; Nagashima *et al.*, 1992, J. Reprod. Dev. 38: 73-78; Prather *et al.*, 1989, *Biol. Reprod. 41*: 414-419; Prather *et al.*, 1990, *Exp. Zool. 255*: 355-358; Saito *et al.*, 1992, *Assis.*

Reprod. Tech. Andro. 259: 257-266; Terlouw *et al.*, 1992, *Theriogenology 37*: 309, Pokajaeva *et al.*, *Nature* 407, 86-90 (2000); Onishi *et al.*, *Science* 289 1188-1190 (2000); and Betthauser *et al.*, *Nature Biotechnology* 18: 1055-1059 (2000).

Researchers have also developed methods for generating transgenic cells, which may be applicable to the production of transgenic animals. Although several viral vectors, non-viral vectors, and other delivery systems have been developed for establishing transgenic cells, many of these technologies are constrained by multiple limitations. Specifically, these limitations include (1) the size of inserted DNA is limited to approximately 10 kilobases (kb); (2) integration of the DNA of interest cannot be specifically targeted into the cell's nuclear DNA; and (3) expression of a recombinant product from the DNA of interest cannot be well controlled. *See, e.g.*, Mitani *et al.*, 1993, *Trends Biotech, 11*: 162-166; U.S. Patent 5,633,067, "Method of Producing a Transgenic Bovine or Transgenic Bovine Embryo," DeBoer *et al.*, issued May 27, 1997; U.S. Patent 5,612,205, "Homologous Recombination in Mammalian Cells," Kay *et al.*, issued March 18, 1997; and PCT publication WO 93/22432, "Method for Identifying Transgenic Pre-Implantation Embryos," all of which are incorporated by reference herein in their entirety, including all figures, drawings, and tables.

Artificial chromosome technology is not constrained by the above-defined limitations. Moreover, researchers have discovered that artificial chromosomes can be replicated *de novo*. *See, e.g.*, Kereso *et al.*, 1996, *Chromosome Research 4*: 226-239, Holló *et al.*, 1996, *Chromosome Research 4*: 240-247, United States Patent No. 6,025,155, and United States Patent No. 6,077,697.

Each reference used to provide background information in this section is hereby incorporated by reference in its entirety, including ant tables, figures, and claims.

Despite progress towards cloning mammals and establishing transgenic cells, there remains a great need in the art for materials and methods that enhance the efficiency for cloning transgenic animals. In particular, there remains a great need in the art to provide pluripotent and totipotent transgenic cells that can be utilized as nuclear donors. Furthermore, there remains a long felt need in the art for providing cell lines that are karyotypically stable and transgenic, which can be utilized in processes for cloning transgenic animals.

SUMMARY OF THE INVENTION

The invention relates in part to transgenic, totipotent, mammalian cells comprising one or more large, heterologous DNA constructs of 100 kbp or more. Preferably, the large DNA construct(s) are artificial chromosomes. Mammalian cells containing the large heterologous DNA construct(s) may be used for producing transgenic embryos and transgenic animals cloned from such cells. The invention is also directed in part to processes for producing totipotent cells that comprise one or more large, heterologous DNA constructs; processes for utilizing such cells; and processes for producing transgenic embryos and transgenic animals cloned from such cells.

Thus, in a first aspect, the invention features a method for producing transgenic cells by inserting a large, heterologous DNA construct of 100 kbp or more into cells. Such cells may preferably be used as nuclear donor cells in methods to produce transgenic animals, most preferably ungulates.

Preferably, a large, heterologous DNA construct is at least 200 Kbp, at least 300 Kbp, at least 400 Kbp, at least 500 Kbp, at least 750 Kbp, at least 1 Mbp, at least 5 Mbp, at least 10 Mbp, at least 20 Mbp, at least 50 Mbp, at least 100 Mbp, at least 500 Mbp, or at least 1000 Mbp. Particularly useful are artificial chromosomes of between 100 Kbp and 500 Mbp; between 500 Kbp and 500 Mbp; and between 1 Mbp and 500 Mbp.

In certain embodiments, the large, heterologous DNA construct(s) of this aspect are artificial chromosomes. Advantages of using artificial chromosomes include: (1) target DNA greater than 10 kb can be inserted into cells; (2) the location of target DNA of interest can be controlled; (3) transgenic animals and embryos containing large foreign genes, or a large copy number of one or more foreign genes, in a majority of cells can be obtained; and (4) the expression levels of a recombinant product from the DNA of interest can be manipulated *in vitro*. Specifically, expression levels can be manipulated by controlling the copy number of target DNA and/or its regulation by promoters, enhancers, *etc.*, in an artificial chromosome, as defined in greater detail hereafter.

The term "artificial chromosome" as used herein refers to nucleic acid molecules that are generated by the manipulation of DNA, contain a centromere, and are capable of stable, autonomous replication in cells. An artificial chromosome (1) can replicate with naturally occurring chromosomes in the nucleus of target cell; (2) can be large in size (ranging in size from 100 kilobase pairs (Kbp) to 1000 megabase pairs (Mbp) in length, or more); (3) typically comprises a centromere, origins of replication, and telomeres; and (4) can comprise neutral DNA. Neutral DNA does not encode products that significantly alter the functions of a cell in which the artificial chromosome is located. For example, neutral DNA may encode ribosomal RNA. It is not typical that increasing levels of ribosomal RNA significantly alters cell functions. Neutral DNA can also be referred to as "satellite DNA."

Preferably, an artificial chromosome is at least 200 Kbp, at least 300 Kbp, at least 400 Kbp, at least 500 Kbp, at least 750 Kbp, at least 1 Mbp, at least 5 Mbp, at least 10 Mbp, at least 20 Mbp, at least 50 Mbp, at least 100 Mbp, at least 500 Mbp, or at least 1000 Mbp. Particularly useful are artificial chromosomes of between 100 Kbp and 500 Mbp; between 500 Kbp and 500 Mbp; and between 1 Mbp and 500 Mbp.

Materials and methods for producing, identifying, and characterizing artificial chromosomes are well known in the art. *See, e.g.*, Kereso *et al.*, 1996, *Chromosome Research 4*: 226-239, Holló *et al.*, 1996, *Chromosome Research 4*: 240-247, International publication nos. WO00/18941, WO98/08964, WO97/16533 and WO97/40183, and United States Patent Nos. 5,721,118, 6,025,155, 6,077,697, and 6,133,503, each of which is incorporated herein by reference in its entirety including all figures, tables, and drawings. These publications also describe shuttle vectors useful for incorporating target DNA into artificial chromosomes. Artificial chromosomes can arise from a portion of a natural chromosome by manipulation. Artificial chromosomes can be detected in cells by using chromosome identification techniques well known in the art. An example of such a technique is chromosome karyotype analysis.

Mammalian artificial chromosomes (MACs) can be generated by cellular mediated chromosome assembly from transfected alphoid, telomeric and marker DNAs (Harrington J.J. et al. Nature Genetics, 15, 345-355, 1997; Ikeno, M. et al, Nature Biotechnology 16, 431-439, 1998; Henning, K.A. et al, PNAS USA 96, 592-597, 1999) and even from non-alphoid DNA (du Sart D, et al, Nature Genetics 16, 144-153, 1997).

Minichromosomes may be generated by fragmenting natural human chromosomes using telomere-directed breakage (Shen MH, et al, Human Molecular Genetics 6, 1375-1382, 1997; Shen MH et al, Current Biology 10, 31-34, 1999). It is possible to transfer human-murine minichromosome chimeras (Shen MH et al, Current Biology 10, 31-34, 1999), fragmented human minichromosomes Tomizuka K et al, Nature Genetics 16, 133-143, 1997; Tomizuka K et al, PNAS USA 97, 722-797, 2000), and human small accessory chromosomes (SACs; Vermeesch JR et al, Human Genetics 105, 611-618, 1999) via microcell-mediated chromosome transfer (MMCT) to recipient cells.

The term "target DNA" as used herein refers to DNA that is intended to be or has been incorporated into a large heterologous DNA construct, preferably an artificial chromosome. The term "heterologous" is defined below. Target DNA can encode multiple types of recombinant products, as defined hereafter, and may exist in multiple copies when introduced into an artificial chromosome. One advantage of artificial chromosome technology is that target DNA copy number can be controlled and monitored in an artificial chromosome *in vitro* before the artificial chromosome comprising the target DNA is introduced into a cell. In addition, depending on the promoter used, expression can also be monitored *in vitro*. This advantage is contrasted with many existing techniques for creating transgenic cells, which cause random insertion of target DNA into a cell nuclear DNA. Materials and methods for introducing target DNA into an artificial chromosome and materials and methods for introducing the resulting artificial chromosome into cells are defined hereafter.

The term "heterologous nucleic acid" refers to nucleic acids having (1) a nucleic acid sequence that differs from the nucleic acid sequences present in cell's naturally occurring nuclear DNA; (2) a subset of nucleic acid having a nucleotide sequence that is present in cell nuclear DNA, but that exists in different proportions in the heterologous nucleic acid than in cell nuclear DNA; (3) a nucleic acid sequence originating from a species other than the species from which cell nuclear DNA originates; and (4) a nucleic acid sequence that differs from the DNA sequences present in cell's naturally occurring mitochondrial DNA.

Artificial chromosomes, such as mammalian artificial chromosomes [MACs], can be generated and isolated by the methods described in the publications above. In

particularly preferred embodiments, two types of artificial chromosomes are used, both of which function in cells as stable, functional chromsomes. One type, herein referred to as ACEs ("Artificial Chromosome Expression systems" based on satellite DNA) is a stable heterochromatic chromosome, and the other type is a *de novo*-formed minichromosomes based on amplification of euchromatin.

Artificial chromosomes, and, in particular the two preferred types discussed above, provide an extra-genomic locus for targeted integration of up to multi-megabase pair size DNA fragments that contain single or multiple genes, including multiple copies of a single gene operatively linked to one promoter or each copy or several copies linked to separate promoters. Thus, methods provided can be used to introduce genes via MACs into cells and tissues of ungulate mammals. The artificial chromosomes with integrated heterologous DNA may be used in methods of production of gene products, particularly products that require expression of multigenic biosynthetic pathways, and also are intended for delivery into the nuclei of cells, such as nuclear donor cells used in nuclear transfer procedures, for production of transgenic ungulate mammals.

Additionally, such artificial chromosomes provide extra-genomic specific integration sites for introduction of genes encoding proteins of interest and permit up to multi-megabase size DNA integration so that, for example, genes encoding an entire metabolic pathway, a very large gene such as the cystic fibrosis transmembrane conductance regulator gene (approximately 250 kb genomic DNA gene), or several genes, such as multiple genes encoding a series of antigens for preparation of a multivalent vaccine, can be stably introduced into a cell.

The artificial chromosomes described herein, including ACEs and euchromatin-based minichromosomes, can be generated by introducing heterologous DNA, preferably including DNA encoding one or multiple selectable marker(s), into cells, preferably a stable cell line, growing the cells under selective conditions, and identifying from among the resulting cell clones those that include chromosomes with more than one centromere, fragments thereof, and/or heterochromatic structures. Amplification that produces the additional centromere(s) occurs in cells that contain chromosomes in which heterologous DNA has integrated near the centromere in the pericentric region of the chromosome. Selected cells comprising intermediates in the

formation of such artificial chromosomes can then be used to generate complete artificial chromosomes.

For example, continued culture of cells containing a formerly dicentric chromosome under conditions that destabilize chromosomes (such as BrdU treatment) and/or under selective conditions can yield ACEs. Similarly, artificial chromosomes can be generated by culturing cells with multi-centric (typically dicentric) chromosomes under conditions whereby the chromosome breaks to form a minichromosome and a formerly dicentric chromosome.

Among the MACs provided herein can be ACEs, which are predominantly heterochromatic (i.e., contain more heterochromatin than euchromatin, and preferably contain about 70% heterochromatin), and can comprise repeating units of short satellite DNA, so that without insertion of heterologous or foreign DNA, the chromosomes preferably contain no genetic information. They can thus be used as "safe" vectors for delivery of DNA to mammalian hosts because they do not contain any potentially harmful genes. ACEs are generated, not from the minichromosome fragment as, for example, in U.S. Pat. No. 5,288,625 (which is incorporated herein by reference in its entirety including all figures, tables, and drawings), but from the fragment of the formerly dicentric chromosome. In addition, euchromatic minichromosomes can be generated. Methods for generating one type of MAC, the minichromosome, is described in U.S. Pat. No. 5,288,625 (which is incorporated herein by reference in its entirety including all figures, tables, and drawings), along with its use for the expression of heterologous DNA are provided.

In preferred embodiments, (1) the artificial chromosome is an ACEs comprising one or more markers; (2) a marker is an antibiotic resistance gene selected from the group consisting of neomycin resistance gene, hygromycin resistance gene, and puromycin resistance gene; (3) the artificial chromosome comprises a DNA sequence that encodes one or more recombinant products; (4) a recombinant product is a ribozyme; (5) a recombinant product is antisense RNA; (6) a recombinant product is a peptide; (7) a recombinant product is a polypeptide; (8) a recombinant product is a protein; (9) a recombinant product is an enzyme; (10) a recombinant product is expressed in a biological fluid; (11) a recombinant product is expressed in a tissue; (12) a recombinant product confers resistance to one or more parasites and/or diseases;

(13) an artificial chromosome comprises one or more regulatory elements; (14) a regulatory element is a promoter element; (15) a promoter element is selected from the group consisting of milk protein promoter, urine protein promoter, blood protein promoter, tear duct protein promoter, synovial protein promoter, mandibular gland protein promoter, casein promoter, β-casein promoter, melanocortin promoter, milk serum protein promoter, α-lactalbumin promoter, whey acid protein promoter, uroplakin promoter, and α-actin promoter; (17) a regulatory element is a repressor element; (18) a regulatory element is an insulator element; and (19) a regulatory element is an enhancer element.

The term "marker" as used herein refers to any DNA sequence that distinguishes a cell comprising an artificial chromosome, or a precursor thereof, from a cell that does not comprise the artificial chromosome or precursor. For example, a marker can be used in the initial steps of generating ACEs, whereby the marker distinguishes a cell containing a foreign nucleic acid from a cell that does not contain the foreign nucleic acid. Multiple types of markers, such as genes encoding green fluorescent protein, antibiotic resistance, β-galactosidase, glutamine synthetase, thymidine kinase, cytosine deaminase, and dihydrofolate reductase are well known in the art. Preferred as markers are DNA sequences that encode a molecule which directly or indirectly inactivates a drug that retards the growth of cells not expressing such a molecule. Examples of these latter described markers are blasticidin-S, neomycin, hygromycin, and puromycin resistance genes. These examples are not meant to be limiting and the invention relates in part to any marker known in the art.

The term "ribozyme" as used herein refers to ribonucleic acid molecules that can cleave other RNA molecules in specific regions. Ribozymes can bind to discrete regions on a RNA molecule, and then specifically cleave a region within that binding region or adjacent to the binding region. Ribozyme techniques can thereby decrease the amount of polypeptide translated from formerly intact message RNA molecules. For specific descriptions of ribozymes, *see* U.S. Patent 5,354,855, entitled "RNA Ribozyme which Cleaves Substrate RNA without Formation of a Covalent Bond," Cech *et al.*, issued on October 11, 1994, and U.S. Patent 5,591,610, entitled "RNA Ribozyme Polymerases, Dephosphorylases, Restriction Endoribonucleases and Methods," Cech *et*

al., issued on January 7, 1997, both of which are incorporated by reference in their entireties including all figures, tables, and drawings.

The term "antisense RNA" as used herein refers to any RNA that binds to mRNA with enough affinity to decrease the amount of protein translated from the mRNA. The amount of protein translated from the mRNA is preferably decreased by more than 20%; more preferably decreased by more than 50%, 70%, and 80%; and most preferably decreased by more than 90%. Antisense RNA materials and methods are well known in the art.

The terms "biological fluid" and the term "tissue" as used herein refer to any fluid or tissue in or from a biological organism. The fluids may include, but are not limited to, tears, saliva, milk, urine, amniotic fluid, semen, plasma, oviductal fluid, and synovial fluid. The tissues may include, but are not limited to, lung, heart, blood, liver, muscle, brain, pancreas, skin, and others.

The term "confers resistance" as used herein refers to the ability of a recombinant product to completely abrogate or partially alleviate the symptoms of a disease or parasitic condition. Hence, if a disease is related to inflammation, for example, a recombinant product can confer resistance to that inflammation if the inflammation decreases upon expression of the recombinant product. A recombinant product may confer resistance or partially confer resistance to a disease or parasitic condition, for example, if the recombinant product is an antisense RNA molecule that specifically binds to an mRNA molecule encoding a polypeptide responsible for the inflammation.

In preferred embodiments, the DNA with the selectable marker that is introduced into cells to generate artificial chromosomes includes sequences that target it to the pericentric region of the chromosome. Integration of the DNA into existing chromosomes in the cells can induce amplification that results in generation of additional centromeres.

Transgenic Cells

Large heterologous nucleic acid constructs, such as artificial chromosomes, can then be introduced into cells to produce stable transformed cell lines and cells. Introduction is effected by any suitable method or combination of methods including,

but not limited to microinjection, cell fusion, microcell fusion, electroporation, sonoporation, electrofusion, projectile bombardment, calcium phosphate precipitation, lipid-mediated transfer systems, ligand/receptor systems and other such methods well known to the skilled artisan. ACEs in particular can be readily isolated and used for gene delivery. These artificial chromosomes can also be used in gene product production systems, production of humanized genetically transformed animal organs, and, most preferably, the generation of transgenic ungulates.

In certain embodiments, the invention relates to transgenic, totipotent, mammalian cells comprising at least one artificial chromosome, but the invention relates in part to any number of artificial chromosomes in a totipotent mammalian cell. A totipotent mammalian cell preferably comprises ten or fewer artificial chromosomes; more preferably comprises six or fewer artificial chromosomes, four or fewer artificial chromosomes, or two or fewer artificial chromosomes; and most preferably comprises one artificial chromosome. If a totipotent mammalian cell of the invention comprises more than one artificial chromosome, the artificial chromosomes may be identical or may differ from one another.

The term "transgenic" as used herein in reference to cells refers to a cell that comprises heterologous nucleic acid, preferably deoxyribonucleic acid (DNA).

In preferred embodiments, a transgenic cell comprises one or more heterologous DNA sequences. In other preferred embodiments, a transgenic cell is a cell in which one or more endogenous genes have been deleted, duplicated, activated, or modified. In particularly preferred embodiments, a transgenic cell comprises both one or more heterologous DNA sequences, and one or more endogenous genes that have been deleted, duplicated, activated, or modified.

An artificial chromosome present in a transgenic cell can comprise heterologous DNA. Heterologous DNA can encode multiple types of recombinant products, as defined hereafter.

The term "transgene" as used herein refers to a single gene that is partially or entirely transgenic in origin. In certain embodiments, greater than 50% of the transgene consists of heterologous DNA. In preferred embodiments, greater than 75% of the transgene consists of heterologous DNA, greater than 80% of the transgene consists of

heterologous DNA, greater than 90% of the transgene consists of heterologous DNA, greater than 95% of the transgene consists of heterologous DNA, greater than 98% of the transgene consists of heterologous DNA, and 100% of the transgene consists of heterologous DNA.

The term "different nucleic acid sequence" as used herein refers to nucleic acid sequences that are not substantially similar. The term "substantially similar" as used herein in reference to nucleic acid sequences refers to two nucleic acid sequences having preferably 80% or more nucleic acid identity, more preferably 90% or more nucleic acid identity or most preferably 95% or more nucleic acid identity. Nucleic acid identity is a property of nucleic acid sequences that measures their similarity or relationship when aligned by means known to one skilled in the art. Identity is measured by dividing the number of identical bases in the two sequences by the total number of bases and multiplying the product by 100. Thus, two copies of exactly the same sequence have 100% identity, while sequences that are less highly conserved and have deletions, additions, or replacements have a lower degree of identity. Those skilled in the art will recognize that several computer programs are available for determining sequence identity and similarity using standard parameters, for example Gapped BLAST or PSI-BLAST (Altschul, *et al.* (1997) Nucleic Acids Res. 25:3389-3402), BLAST (Altschul, *et al.* (1990) J. Mol. Biol. 215:403-410), and Smith-Waterman (Smith, et al. (1981) J. Mol. Biol. 147:195-197). Preferably, the default settings of these programs will be employed, but those skilled in the art recognize whether these settings need to be changed and know how to make the changes.

The term "substantially similar" as used herein in reference to amino acid sequences refers to two amino acid sequences having preferably 50% or more amino acid identity, more preferably 70% or more amino acid identity or most preferably 90% or more amino acid identity. Amino acid identity is a property of amino acid sequence that measures their similarity or relationship. Identity is measured by dividing the number of identical residues in the two sequences by the total number of residues and multiplying the product by 100. Thus, two copies of exactly the same sequence have 100% identity, while sequences that are less highly conserved and have deletions, additions, or replacements have a lower degree of identity.

"Similarity" in protein sequences is measured by dividing the number of identical residues plus the number of conservatively substituted residues (see Bowie, *et al.* Science, (1999) 247, 1306-1310, which is incorporated herein by reference in its entirety, including any drawings, figures, or tables) by the total number of residues and gaps and multiplying the product by 100. "Similarity" in nucleic acid sequences is measured by dividing the number of identical bases by the total number of residues and gaps and multiplying the product by 100.

The term "recombinant product" as used herein refers to the product produced from a target DNA sequence. A recombinant product can be a peptide, a polypeptide, a protein, an enzyme, an antibody, an antibody fragment, a polypeptide that binds to a regulatory element (a term described hereafter), a structural protein, an RNA molecule, and/or a ribozyme, for example. These products are well defined in the art. This list of products is for illustrative purposes only and the invention relates to other types of recombinant products.

In preferred embodiments, (1) the mammalian cell is an ungulate cell; (2) the ungulate is selected from the group consisting of bovids, ovids, cervids, suids, equids and camelids; (3) the ungulate is bovine; (4) the mammalian cell is a nonembryonic cell; (5) the mammalian cell is a fetal cell; and (6) the mammalian cell is an adult cell.

The term "mammalian" as used herein refers to any animal of the class *Mammalia*. Preferably, a mammalian cell or cell line is a placental, a monotreme and a marsupial. Most preferably, a mammalian cell or cell line is a canid, felid, murid, leporid, ursid, mustelid, ungulate, ovid, suid, equid, bovid, caprid, cervid, and a human or non-human primate. A mammalian cell or cell line can be isolated from any source of mammalian cells including, but not limited to, a mammalian embryo, a mammalian fetus, and a mammalian animal.

The term "canid" as used herein refers to any animal of the family *Canidae*. Preferably, a canid cell or cell line is isolated from a wolf, a jackal, a fox, and a domestic dog.

The term "felid" as used herein refers to any animal of the family *Felidae*. Preferably, a felid cell or cell line is isolated from a lion, a tiger, a leopard, a cheetah, a cougar, and a domestic cat.

The term "murid" as used herein refers to any animal of the family *Muridae*. Preferably, a murid cell or cell line is isolated from a mouse and a rat.

The term "leporid" as used herein refers to any animal of the family *Leporidae*. Preferably, a leporid cell or cell line is isolated from a rabbit.

The term "ursid" as used herein refers to any animal of the family *Ursidae*. Preferably, a ursid cell or cell line is isolated from a bear.

The term "mustelid" as used herein refers to any animal of the family *Mustelidae*. Preferably, a mustelid cell or cell line is isolated from a weasel, a ferret, an otter, a mink, and a skunk.

The term "primate" as used herein refers to any animal of the *Primate* order. Preferably, a primate cell or cell line is isolated from an ape, a monkey, a chimpanzee, and a lemur.

The term "ungulate" as used herein refers to any animal of the polyphyletic group formerly known as the taxon *Ungulata*. Preferably, an ungulate cell or cell line is isolated from a camel, a hippopotamus, a horse, a tapir, and an elephant. Most preferably, an ungulate cell or cell line is isolated from a sheep, a cow, a goat, and a pig. Especially preferred in the bovine species are *Bos taurus*, *Bos indicus*, and *Bos buffaloes* cows or bulls.

The term "ovid" as used herein refers to any animal of the family *Ovidae*. Preferably, an ovid cell or cell line is isolated from a sheep.

The term "suid" as used herein refers to any animal of the family *Suidae*. Preferably, a suid cell or cell line is isolated from a pig or a boar.

The term "equid" as used herein refers to any animal of the family *Equidae*. Preferably, an equid cell or cell line is isolated from a zebra or an ass. Most preferably, an equid cell or cell line is isolated from a horse.

The term "bovid" as used herein refers to any animal of the family *Bovidae*. Preferably, an bovid cell or cell line is isolated from an antelope, an oxen, a cow, and a bison.

The term "caprid" as used herein refers to any animal of the family *Caprinae*. Preferably, a caprid cell or cell line is isolated from a goat.

The term "cervid" as used herein refers to any animal of the family *Cervidae*. Preferably, a cervid cell or cell line is isolated from a deer.

The term "immortalized" or "permanent" as used herein in reference to cells refers to cells that have exceeded the Hayflick limit. The Hayflick limit can be defined as the number of cell divisions that occur before a cell line becomes senescent. Hayflick set this limit to approximately 60 divisions for most non-immortalized cells. *See, e.g.*, Hayflick and Moorhead, 1961, *Exp. Cell. Res. 25*: 585-621; and Hayflick, 1965, *Exp. Cell Research 37*: 614-636, incorporated herein by reference in their entireties including all figures, tables, and drawings. Therefore, an immortalized cell line can be distinguished from non-immortalized cell lines if the cells in the cell line are able to undergo more than 60 divisions. If the cells of a cell line are able to undergo more than 60 cell divisions, the cell line is an immortalized or permanent cell line. The immortalized cells of the invention are preferably able to undergo more than 70 divisions, are more preferably able to undergo more than 80 divisions, and are most preferably able to undergo more than 90 cell divisions.

The terms "primary culture" and "primary cell" refer to cells taken from a tissue source, and their progeny, grown in culture before subdivision and transfer to a subculture.

The terms "plated" or "plating" as used herein in reference to cells refer to establishing cell cultures *in vitro*. For example, cells can be diluted in cell culture media and then added to a cell culture plate or cell culture dish. Cell culture plates are commonly known to a person of ordinary skill in the art. Cells may be plated at a variety of concentrations and/or cell densities. In preferred embodiments, plated cells may grow to confluence.

The meaning of the term "cell plating" can also extend to the term "cell passaging." Cells of the invention can be passaged using cell culture techniques well known to those skilled in the art. The term "cell passaging" refers to such techniques which typically involve the steps of (1) releasing cells from a solid support and disassociation of these cells, and (2) diluting the cells in fresh media suitable for cell

proliferation. Immortalized cells can be successfully grown by plating the cells in conditions where they lack cell to cell contact. Cell passaging may also refer to removing a portion of liquid medium bathing cultured cells and adding liquid medium from another source to the cell culture to dilute the cell concentration.

The term "proliferation" as used herein in reference to cells refers to a group of cells that can increase in size and/or can increase in numbers over a period of time.

The term "confluence" as used herein refers to a group of cells where a large percentage of the cells are physically contacted with at least one other cell in that group. Confluence may also be defined as a group of cells that grow to a maximum cell density in the conditions provided. For example, if a group of cells can proliferate in a monolayer and they are placed in a culture vessel in a suitable growth medium, they are confluent when the monolayer has spread across a significant surface area of the culture vessel. The surface area covered by the cells preferably represents about 50% of the total surface area, more preferably represents about 70% of the total surface area, and most preferably represents about 90% of the total surface area.

The cells and cell lines of the instant invention may be cultured. The term "cultured" as used herein in reference to cells refers to one or more cells that are undergoing cell division or not undergoing cell division in an *in vitro* environment. An *in vitro* environment can be any medium known in the art that is suitable for maintaining cells *in vitro*, such as suitable liquid media or agar, for example. Specific examples of suitable *in vitro* environments for cell cultures are described in *Culture of Animal Cells: a manual of basic techniques* (3rd edition), 1994, R.I. Freshney (ed.), Wiley-Liss, Inc.; *Cells*: a laboratory manual (vol. 1), 1998, D.L. Spector, R.D. Goldman, L.A. Leinwand (eds.), Cold Spring Harbor Laboratory Press; and *Animal Cells: culture and media*, 1994, D.C. Darling, S.J. MorganJohn Wiley and Sons, Ltd., each of which is incorporated herein by reference in its entirety including all figures, tables, and drawings. Cells may be cultured in suspension and/or in monolayers with one or more substantially similar cells. Cells may be cultured in suspension and/or in monolayers with a heterogeneous population of cells. The term "heterogeneous" as utilized in the previous sentence can relate to any cell characteristics, such as cell type and cell cycle stage, for example. Cells may be cultured in suspension, cultured as monolayers attached to a solid support, and/or cultured on a layer of feeder cells, for

example. The term "feeder cells" is defined hereafter. Furthermore, cells may be successfully cultured by plating the cells in conditions where they lack cell to cell contact. Preferably, cultured cells undergo cell division and are cultured for at least 5 days, more preferably for at least 10 days or 20 days, and most preferably for at least 30 days. Preferably, a significant number of cultured cells do not terminate while in culture. The terms "terminate" and "significant number" are defined hereafter. Nearly any type of cell can be placed in cell culture conditions. Cultured cells can be utilized to establish a cell line.

In particularly preferred embodiments, a cell may be "clonally propagated." In these embodiments, cells are diluted to an extent such that, statistically, some or all of the culture vessels into which the diluted cells are placed will contain only a single cell. Thus, the culture that grows within these culture vessels will be derived from a single cell. Materials and methods for clonally propagating cells are described in U.S. Patent Application No. 09/753,323 (attorney docket number 030653.0026.CIP1, filed December 28, 2000), which is hereby incorporated in its entirety.

The term "terminating" and "terminate" as used herein with regard to cultured cells may refer to cells that undergo cell death, which can be measured using multiple techniques known to those skilled in the art (*e.g.*, CytoTox96® Cytotoxicity Assay, Promega, Inc. catalog no. G1780; Celltiter96® Aqueous Cell Proliferation Assay Kit, Promega, Inc. catalog no. G3580; and Trypan Blue solution for cytotoxicity assays, Sigma catalog no. T6146). Termination may also be a result of apoptosis, which can be measured using multiple techniques known to persons skilled in the art (*e.g.*, Dead End™ Apoptosis Detection Kit, Promega, Inc. catalog no. G7130). Terminated cells may be identified as those that have undergone cell death and/or apoptosis and have released from a solid surface in culture. In addition, terminated cells may lack intact membranes which can be identified by procedures described above. Also, terminated cells may exhibit decreased metabolic activity, which may be caused in part by decreased mitochondrial activity that can be identified by rhodamine 1,2,3, for example. Furthermore, termination can be refer to cell cultures where a significant number of cultured cells terminate. The term "significant number" in the preceding sentence refers to about 80% of the cells in culture, preferably about 90% of the cells in

culture, more preferably about 100% of the cells in culture, and most preferably 100% of the cells in culture.

The term "suspension" as used herein refers to cell culture conditions in which the cells are not attached to a solid support. Cells proliferating in suspension can be stirred while proliferating using apparatus well known to those skilled in the art.

The term "monolayer" as used herein refers to cells that are attached to a solid support while proliferating in suitable culture conditions. A small portion of the cells proliferating in the monolayer under suitable growth conditions may be attached to cells in the monolayer but not to the solid support. Preferably less than 15% of these cells are not attached to the solid support, more preferably less than 10% of these cells are not attached to the solid support, and most preferably less than 5% of these cells are not attached to the solid support.

The term "substantially similar" as used herein in reference to mammalian cells refers to cells from the same organism and the same tissue. Substantially similar can also refer to cell populations that have not significantly differentiated. For example, preferably less than 15% of the cells in a population of cells have differentiated, more preferably less than 10% of the cell population have differentiated, and most preferably less than 5% of the cell population have differentiated.

The term "cell line" as used herein refers to cultured cells that can be passaged one or more times. The invention preferably relates to cell lines that can be passaged more than 2, 5, 10, 15, 20, 30, 50, 80, 100, and 200 times. The concept of cell passaging is defined previously.

In preferred embodiments, (1) the mammalian cell is subject to manipulation; (2) the manipulation comprises the step of nuclear transfer; (3) the nuclear transfer comprises the step of inserting the totipotent mammalian cell into a recipient oocyte; (4) the manipulation comprises a step of cryopreservation of the mammalian cell; (5) the manipulation comprises a step of thawing of the mammalian cell; (6) the manipulation comprises a step of culturing the mammalian cell; (7) the manipulation comprises a step of passaging the mammalian cell; (8) the manipulation comprises a step of synchronizing the mammalian cell; (9) the manipulation comprises a step of

introducing the mammalian cell to feeder cells; and (10) the manipulation comprises a step of dissociating the mammalian cell from other cells.

The term "manipulation" as used herein refers to the common usage of the term, which is the management or handling directed towards some object. Examples of manipulations are described herein.

The term "thawing" as used herein refers to the process of increasing the temperature of a cryopreserved cell, embryo, or portions of animals. Methods of thawing cryopreserved materials such that they are active after the thawing process are well-known to those of ordinary skill in the art.

The term "dissociating" as used herein refers to the materials and methods useful for pulling a cell away from another cell. For example, a blastomere (*i.e.*, a cellular member of a morula or blastocyst stage embryo) can be pulled away from the rest of the developing cell mass by techniques and apparatus well known to a person of ordinary skill in the art. *See, e.g.*, U.S. Patent 4,994,384, entitled "Multiplying Bovine Embryos," issued on February 19, 1991, hereby incorporated herein by reference in its entirety, including all figures, tables, and drawings. Alternatively, cells proliferating in culture can be separated from one another to facilitate such processes as cell passaging, which is described previously. In addition, dissociation of a cultured cell from a group of cultured cells can be useful as a first step in the process of nuclear transfer, as described hereafter. When a cell is dissociated from an embryo, the dissociation manipulation can be useful for such processes as re-cloning, a process described herein, as well as a step for multiplying the number of embryos.

The term "non-embryonic cell" as used herein refers to a cell that is not isolated from an embryo. Non-embryonic cells can be differentiated or non-differentiated. Non-embryonic cells refers to nearly any somatic cell, such as cells isolated from an *ex utero* animal. These examples are not meant to be limiting.

The term "fetus" as used herein refers to a developing cell mass that has implanted into the uterine membrane of a maternal host. A fetus can include such defining features as a genital ridge, for example. A genital ridge is a feature easily identified by a person of ordinary skill in the art, and is a recognizable feature in fetuses of most animal species. The term "fetal cell" as used herein refers to any cell isolated

from and/or has arisen from a fetus or derived from a fetus. The term "non-fetal cell" is a cell that is not derived or isolated from a fetus.

When cells are isolated from a fetus, such cells are preferably isolated from fetuses where the fetus is between 20 days and parturition, between 30 days and 100 days, more preferably between 35 days and 70 days and between 40 days and 60 days, and most preferably about a 55 day fetus. An age of a fetus can be determined from the time that an embryo, which develops into the fetus, is established. The term "about" with respect to fetuses refers to plus or minus five days.

The term "parturition" as used herein refers to a time that a fetus is delivered from female recipient. A fetus can be delivered from a female recipient by abortion, c-section, or birth.

In preferred embodiments, the cells and cell lines of the instant invention are primary cells, embryonic cells, non-embryonic cells, fetal cells, genital ridge cells, primordial germ cells, embryonic germ cells, embryonic stem cells, somatic cells, adult cells, fibroblasts, differentiated cells, undifferentiated cells, amniotic cells, ovarian follicular cells, and cumulus cells. Preferably, such cells grow to confluent monolayers in culture.

The term "primordial germ cell" as used herein refers to a diploid somatic cell capable of becoming a germ cell. Primordial germ cells can be isolated from the genital ridge of a developing cell mass. The genital ridge is a section of a developing cell mass that is well-known to a person of ordinary skill in the art. *See, e.g.*, Strelchenko, 1996, *Theriogenology 45*: 130-141 and Lavoir 1994, *J. Reprod. Dev. 37*: 413-424. Such cells, when cultured, are referred to by the skilled artisan as "embryonic germ cells."

The term "embryonic stem cell" as used herein refers to pluripotent cells isolated from an embryo that are maintained in *in vitro* cell culture. Embryonic stem cells may be cultured with or without feeder cells. Embryonic stem cells can be established from embryonic cells isolated from embryos at any stage of development, including blastocyst stage embryos and pre-blastocyst stage embryos. Embryonic stem cells are well known to a person of ordinary skill in the art. *See, e.g.*, WO 97/37009, entitled "Cultured Inner Cell Mass Cell-Lines Derived from Ungulate Embryos," Stice & Golueke, published October 9, 1997, and Yang & Anderson, 1992, *Theriogenology*

38: 315-335, both of which are incorporated herein by reference in their entireties, including all figures, tables, and drawings.

The term "differentiated cell" as used herein refers to a cell that has developed from an unspecialized phenotype to that of a specialized phenotype. For example, embryonic cells can differentiate into an epithelial cell lining the intestine. It is highly unlikely that differentiated cells revert into their precursor cells *in vivo* or *in vitro*. However, materials and methods of the invention can reprogram differentiated cells into immortalized, totipotent cells. Differentiated cells can be isolated from a fetus or a live born animal, for example.

The term "undifferentiated cell" as used herein refers to a cell that has an unspecialized phenotype and is capable of differentiating. An example of an undifferentiated cell is a stem cell.

The term "asynchronous population" as used herein refers to cells that are not arrested at any one stage of the cell cycle. Many cells can progress through the cell cycle and do not arrest at any one stage, while some cells can become arrested at one stage of the cell cycle for a period of time. Some known stages of the cell cycle are G_0, G_1, S, G_2, and M. An asynchronous population of cells is not manipulated to synchronize into any one or predominantly into any one of these phases. Cells can be arrested in the G_0 stage of the cell cycle, for example, by utilizing multiple techniques known in the art, such as by serum deprivation. Examples of methods for arresting non-immortalized cells in one part of the cell cycle are discussed in WO 97/07669, entitled "Quiescent Cell Populations for Nuclear Transfer," hereby incorporated herein by reference in its entirety, including all figures, tables, and drawings.

The terms "synchronous population" and "synchronizing" as used herein refer to a fraction of cells in a population that are arrested (*i.e.*, the cells are not dividing) in a discrete stage of the cell cycle. Preferably, about 50% of the cells in a population of cells are arrested in one stage of the cell cycle, more preferably about 70% of the cells in a population of cells are arrested in one stage of the cell cycle, and most preferably about 90% of the cells in a population of cells are arrested in one stage of the cell cycle. Cell cycle stage can be distinguished by relative cell size as well as by a variety of cell markers well known to a person of ordinary skill in the art. For example, cells can be distinguished by such markers by using flow cytometry techniques well known to a

person of ordinary skill in the art. Alternatively, cells can be distinguished by size utilizing techniques well known to a person of ordinary skill in the art, such as by the utilization of a light microscope and a micrometer, for example.

The term "adult cell" as used herein refers to a cell from a live-born animal.

The term "amniotic cell" as used herein refers to any cultured or non-cultured cell isolated from amniotic fluid. Examples of methods for isolating and culturing amniotic cells are discussed in Bellow *et al.*, 1996, *Theriogenology 45*: 225; Garcia & Salaheddine, 1997, *Theriogenology 47*: 1003-1008; Leibo & Rail, 1990, *Theriogenology 33*: 531-552; and Vos *et al.*, 1990, *Vet. Rec. 127*: 502-504, each of which is incorporated herein by reference in its entirety, including all figures tables and drawings. Particularly preferred are cultured amniotic cells that are rounded (*e.g.*, cultured amniotic cells that do not display a fibroblast-like morphology). Also preferred amniotic cells are fetal fibroblast cells. The terms "fibroblast," fibroblast-like," "fetal," and "fetal fibroblast" are defined hereafter.

The term "fibroblast" as used herein refers to cultured cells having a flattened and elongated morphology that are able to grow in monolayers. Preferably, fibroblasts grow to confluent monolayers in culture. While fibroblasts characteristically have a flattened appearance when cultured on culture media plates, fetal fibroblast cells can also have a spindle-like morphology. Fetal fibroblasts may require density limitation for growth, may generate type I collagen, and may have a finite life span in culture of approximately fifty generations. Preferably, fetal fibroblast cells rigidly maintain a diploid chromosomal content. For a description of fibroblast cells, *see, e.g.*, *Culture of Animal Cells: a manual of basic techniques* (3rd edition), 1994, R.I. Freshney (ed), Wiley-Liss, Inc., incorporated herein by reference in its entirety, including all figures, tables, and drawings.

The term "uterine cell" as used herein refers to any cell isolated from a uterus. Preferably, a uterine cell is a cell deriving from a pregnant adult animal. In preferred embodiments, uterine cells are cells obtained from fluid that fills the uterine cavity. Such cells can be obtained by numerous methods well known in the art such as amniocentesis.

The term "ovarian follicular cell" as used herein refers to a cultured or non-cultured cell obtained from an ovarian follicle, other than an oocyte. Follicular cells may be isolated from ovarian follicles at any stage of development, including primordial follicles, primary follicles, secondary follicles, growing follicles, vesicular follicles, maturing follicles, mature follicles, and graafian follicles. Furthermore, follicular cells may be isolated when an oocyte in an ovarian follicle is immature (i.e., an oocyte that has not progressed to metaphase II) or when an oocyte in an ovarian follicle is mature (i.e., an oocyte that has progressed to metaphase II or a later stage of development). Preferred follicular cells include, but are not limited to, pregranulosa cells, granulosa cells, theca cells, columnar cells, stroma cells, theca interna cells, theca externa cells, mural granulosa cells, luteal cells, and corona radiata cells. Particularly preferred follicular cells are cumulus cells. Various types of follicular cells are known and can be readily distinguished by those skilled in the art. *See, e.g., Laboratory Production of Cattle Embryos*, 1994, Ian Gordon, CAB International; *Anatomy and Physiology of Farm Animals* (5th ed.), 1992, R.D. Frandson and T.L. Spurgeon, Lea & Febiger, each of which is incorporated herein by reference in its entirety including all figures, drawings, and tables. Individual types of follicular cells may be cultured separately, or a mixture of types may be cultured together.

The term "cumulus cell" as used herein refers to any cultured or non-cultured cell isolated from cells and/or tissue surrounding an oocyte. Persons skilled in the art can readily identify cumulus cells. Examples of methods for isolating and/or culturing cumulus cells are discussed in Damiani *et al.*, 1996, *Mol. Reprod. Dev. 45*: 521-534; Long *et al.*, 1994, *J. Reprod. Fert. 102*: 361-369; and Wakayama *et al.*, 1998, *Nature 394*: 369-373, each of which is incorporated herein by reference in its entireties, including all figures, tables, and drawings. Cumulus cells may be isolated from ovarian follicles at any stage of development, including primordial follicles, primary follicles, secondary follicles, growing follicles, vesicular follicles, maturing follicles, mature follicles, and graafian follicles. Cumulus cells may be isolated from oocytes in a number of manners well known to a person of ordinary skill in the art. For example, cumulus cells can be separated from oocytes by pipeting the cumulus cell/oocyte complex through a small bore pipette, by exposure to hyaluronidase, or by mechanically disrupting (e.g. vortexing) the cumulus cell/oocyte complex. Additionally, exposure to Ca^{++}/Mg^{++} free media can remove cumulus from immature

oocytes. Also, cumulus cell cultures can be established by placing matured oocytes in cell culture media. Once cumulus cells are removed from media containing increased LH/FSH concentrations, they can to attach to the culture plate.

In a preferred embodiment, the culturing process can comprise the step of selecting totipotent mammalian cells comprising at least one artificial chromosome.

The term "selection" as used herein refers to a process for identifying cells that comprise a large heterologous nucleic acid construct, such as an artificial chromosome. Selection can be effected by identifying a marker region incorporated in an artificial chromosome. The term "marker" is defined previously. Preferably, from 50% to 100% of cells in cell cultures that have undergone selection comprise an artificial chromosome. In particularly preferred embodiments, greater than or equal to 50% of cells in cell cultures that have undergone selection comprise an artificial chromosome. More preferably, greater than or equal to 75% of cells in cell cultures that have undergone selection comprise an artificial chromosome. Most preferably, greater than or equal to 90% of cells in cell cultures that have undergone selection comprise an artificial chromosome.

The term "feeder cells" as used herein refers to cells grown in co-culture with target cells. Target cells can be precursor cells and totipotent cells, for example. Feeder cells can provide, for example, peptides, polypeptides, electrical signals, organic molecules (*e.g.*, steroids), nucleic acid molecules, growth factors (*e.g.*, bFGF), other factors (*e.g.*, cytokines such as LIF and steel factor), and metabolic nutrients to target cells. Certain cells, such as immortalized, totipotent cells may not require feeder cells for healthy growth. Feeder cells preferably grow in a mono-layer.

Feeder cells can be established from multiple cell types. Examples of these cell types are fetal cells, mouse cells, Buffalo rat liver cells, and oviductal cells. These examples are not meant to be limiting. Tissue samples can be broken down to establish a feeder cell line by methods well known in the art (*e.g.*, by using a blender). Feeder cells may originate from the same or different animal species as the precursor cells. In an example of feeder cells established from fetal cells, ungulate fetuses and preferably bovine fetuses may be utilized to establish a feeder cell line where one or more cell types have been removed from the fetus (*e.g.*, primordial germ cells, cells in the head region, and cells in the body cavity region). When an entire fetus is utilized to establish

a fetal feeder cell line, feeder cells (*e.g.*, fibroblast cells) and precursor cells (*e.g.*, primordial germ cells) can arise from the same source (*e.g.*, one fetus).

The term "drug" as used herein refers to any type of molecule that retards the normal growth rate of a cell. A normal cell growth rate is measured in the absence of drug. A drug may also lyse cells.

In another aspect, the invention features a method for producing transgenic ungulates by introducing a large heterologous nucleic acid construct into a nuclear donor cell, then fusing this nuclear donor cell into an enucleated recipient cell to form a nuclear transfer embryo, activating this embryo, and finally transferring this embryo into a maternal host to produce a transgenic animal. In particularly preferred embodiments, the transgenic animal is an ungulate.

Nuclear Transfer

Most preferably, transgenic animals are prepared by introducing a heterologous nucleic acid molecule, preferably an artificial chromosome, into a nuclear donor cell, then fusing this nuclear donor cell into an enucleated recipient cell, most preferably an enucleated oocyte, to form a nuclear transfer embryo, activating this embryo, and finally transferring this embryo into a maternal host to produce a transgenic animal.

In preferred embodiments, the artificial chromosome(s) is introduced into the cybrid by introduction into the nuclear donor cell prior to the fusion with the enucleated recipient cell or enucleated oocyte. In other preferred embodiments, the artificial chromosome(s) is introduced into the cybrid formed by fusion of the nuclear donor cell with the enucleated recipient cell or enucleated oocyte. In yet other preferred embodiments, the artificial chromosome(s) is introduced into the cybrid simultaneously with the fusion of the nuclear donor cell with the enucleated recipient cell or enucleated oocyte

The terms "nuclear transfer" and "nuclear transfer procedure" as used herein refer to introducing a full complement of nuclear DNA from one cell to an enucleated cell. Nuclear transfer methods are well known to a person of ordinary skill in the art. *See,* U.S. Patent No. 4,994,384 to Prather *et al.*, entitled "Multiplying Bovine Embryos," issued on February 19, 1991; U.S. Patent No. 5,057,420 to Massey, entitled "Bovine Nuclear Transplantation," issued on October 15, 1991; U.S. Patent

No. 5,994,619, issued on November 30, 1999 to Stice *et al.*, entitled "Production of Chimeric Bovine or Porcine Animals Using Cultured Inner Cell Mass Cells; U.K. Patents Nos. GB 2,318,578 GB 2,331,751, issued on January 19, 2000 to Campbell *et al.* and Wilmut *et al.*, respectively, entitled "Quiescent Cell Populations For Nuclear Transfer"; U.S. Patent No. 6,011,197 to Strelchenko *et al.*, entitled "Method of Cloning Bovines Using Reprogrammed Non-Embryonic Bovine Cells," issued on January 4, 2000; and in U.S. Patent Application No. 09/753,323 entitled "Method of Cloning Porcine Animals (attorney docket number 030653.0026.CIP1, filed December 28, 2000), each of which are hereby incorporated by reference in its entirety including all figures, tables and drawings. Nuclear transfer may be accomplished by using oocytes that are not surrounded by a zona pellucida.

In a nuclear transfer procedure, a nuclear donor cell, or the nucleus thereof, is introduced into a recipient cell. A recipient cell is preferably an oocyte and is preferably enucleated. However, the invention relates in part to nuclear transfer, where a nucleus of an oocyte is not physically extracted from the oocyte. It is possible to establish a nuclear transfer embryo where nuclear DNA from the donor cell is replicated during cellular divisions. *See, e.g.,* Wagoner *et al.*, 1996, "Functional enucleation of bovine oocytes: effects of centrifugation and ultraviolet light," Theriogenology 46: 279-284. In addition, nuclear transfer may be accomplished by combining one nuclear donor and more than one enucleated oocyte. Also, nuclear transfer may be accomplished by combining one nuclear donor, one or more enucleated oocytes, and the cytoplasm of one or more enucleated oocytes. The resulting combination of a nuclear donor cell and a recipient cell can be referred to variously as a "nuclear transfer embryo," a "hybrid cell," or a "cybrid."

Furthermore, a nuclear donor may arise from an animal of the same species from which a nuclear recipient is isolated. Alternatively, a nuclear donor may arise from an animal of a different specie from which a nuclear recipient is isolated. For example, a differentiated cell isolated from an ear punch of a water buffalo may be utilized as a nuclear donor and an oocyte isolated from a bovine animal may be utilized as a nuclear acceptor. Thus, xenospecific nuclear transfer is contemplated by the instant invention.

The term "nuclear donor" as used herein refers to any cell, or nucleus thereof, having nuclear DNA that can be translocated into an oocyte. A nuclear donor may be a nucleus that has been isolated from a cell. Multiple techniques are available to a person of ordinary skill in the art for isolating a nucleus from a cell and then utilizing the nucleus as a nuclear donor. *See, e.g.*, U.S. Patents Nos. 4,664,097, 6,011,197, and 6,107,543, each of which is hereby incorporated by reference in its entirety including all figures, tables and drawings. Any type of cell can serve as a nuclear donor. Examples of nuclear donor cells include, but are not limited to, cultured and non-cultured cells isolated from an embryo arising from the union of two gametes in vitro or in vivo; embryonic stem cells (ES cells) arising from cultured embryonic cells (*e.g.*, pre-blastocyst cells and inner cell mass cells); cultured and non-cultured cells arising from inner cell mass cells isolated from embryos; cultured and non-cultured pre-blastocyst cells; cultured and non-cultured fetal cells; cultured and non-cultured adult cells; cultured and non-cultured primordial germ cells; cultured and non-cultured germ cells (*e.g.*, embryonic germ cells); cultured and non-cultured somatic cells isolated from an animal; cultured and non-cultured cumulus cells; cultured and non-cultured amniotic cells; cultured and non-cultured fetal fibroblast cells; cultured and non-cultured genital ridge cells; cultured and non-cultured differentiated cells; cultured and non-cultured cells in a synchronous population; cultured and non-cultured cells in an asynchronous population; cultured and non-cultured serum-starved cells; cultured and non-cultured permanent cells; and cultured and non-cultured totipotent cells. *See, e.g.*, Piedrahita *et al.*, 1998, *Biol. Reprod. 58*: 1321-1329; Shim *et al.*, 1997, *Biol. Reprod. 57*: 1089-1095; Tsung *et al.*, 1995, *Shih Yen Sheng Wu Hsueh Pao 28*: 173-189; and Wheeler, 1994, *Reprod. Fertil. Dev. 6*: 563-568, each of which is incorporated herein by reference in its entirety including all figures, drawings, and tables. In addition, a nuclear donor may be a cell that was previously frozen or cryopreserved.

The term "activation" refers to any materials and methods useful for stimulating a cell to divide before, during, and after a nuclear transfer step. Cybrids may require stimulation in order to divide after a nuclear transfer has occurred. The invention pertains to any activation materials and methods known to a person of ordinary skill in the art. Although electrical pulses are sometimes sufficient for stimulating activation of cybrids, other means are sometimes useful or necessary for proper activation of the

cybrid. Chemical materials and methods useful for activating embryos are described below in other preferred embodiments of the invention.

Examples of non-electrical means for activation include agents such as ethanol; inositol trisphosphate (IP_3); Ca^{++} ionophores (*e.g.*, ionomycin) and protein kinase inhibitors (*e.g.*, 6-dimethylaminopurine (DMAP)) ; temperature change; protein synthesis inhibitors (*e.g.*, cyclohexamide); phorbol esters such as phorbol 12-myristate 13-acetate (PMA); mechanical techniques; and thapsigargin. The invention includes any activation techniques known in the art. *See, e.g.*, U.S. Patent No. 5,496,720, entitled "Parthenogenic Oocyte Activation" to Susko-Parrish *et al.*, issued on March 5, 1996; and U.S. Patent No. 6,077,710 , issued on June 20, 2000, each of which is incorporated by reference herein in its entirety, including all figures, tables, and drawings.

The term "fusion" as used herein in reference to nuclear transfer refers to the combination of portions of lipid membranes corresponding to the nuclear donor and the recipient oocyte. Lipid membranes can correspond to the plasma membranes of cells or nuclear membranes, for example. The fusion can occur between the nuclear donor and recipient oocyte when they are placed adjacent to one another, or when the nuclear donor is placed in the perivitelline space of the recipient oocyte, for example. Specific examples for translocation of the totipotent mammalian cell into the oocyte are described hereafter in other preferred embodiments. These techniques for translocation are fully described in the references cited previously herein in reference to nuclear transfer.

The term "electrical pulses" as used herein refers to subjecting the nuclear donor and recipient oocyte to electric current. For nuclear transfer, the nuclear donor and recipient oocyte can be aligned between electrodes and subjected to electrical current. The electrical current can be alternating current or direct current. The electrical current can be delivered to cells for a variety of different times as one pulse or as multiple pulses. The cells are typically cultured in a suitable medium for the delivery of electrical pulses. Examples of electrical pulse conditions utilized for nuclear transfer are described in the references and patents previously cited herein in reference to nuclear transfer.

The term "fusion agent" as used herein in reference to nuclear transfer refers to any compound or biological organism that can increase the probability that portions of plasma membranes from different cells will fuse when a totipotent mammalian cell nuclear donor is placed adjacent to the recipient oocyte. In preferred embodiments fusion agents are selected from the group consisting of polyethylene glycol (PEG), trypsin, dimethylsulfoxide (DMSO), lectins, agglutinin, viruses, and Sendai virus. These examples are not meant to be limiting and other fusion agents known in the art are applicable and included herein.

The term "suitable concentration" as used herein in reference to fusion agents, refers to any concentration of a fusion agent that affords a measurable amount of fusion. Fusion can be measured by multiple techniques well known to a person of ordinary skill in the art, such as by utilizing a light microscope, dyes, and fluorescent lipids, for example.

The term "totipotent" as used herein refers to a cell, embryo, or fetus capable of giving rise to a live born animal. The term "totipotent" can also refer to a cell that gives rise to all of the cells in a particular animal. A totipotent cell can give rise to all of the cells of an animal when it is utilized in a procedure for developing an embryo from one or more nuclear transfer steps. Totipotent cells, embryos, and fetuses may also be used to generate incomplete animals such as those useful for organ harvesting, *e.g.*, having genetic modifications to eliminate growth of an organ or appendage by manipulation of a homeotic gene.

The term "live born" as used herein preferably refers to an animal that exists *ex utero*. A "live born" animal may be an animal that is alive for at least one second from the time it exits the maternal host. A "live born" animal may not require the circulatory system of an *in utero* environment for survival. A "live born" animal may be an ambulatory animal. Such animals can include pre- and post-pubertal animals. As discussed previously, a live born animal may lack a portion of what exists in a normal animal of its kind.

In preferred embodiments, (1) totipotent cells arise from at least one precursor cell; (2) a precursor cell is isolated from and/or arises from any region of a ungulate animal; (3) a precursor cell is isolated from and/or arises from any cell in culture; (4) a precursor cell is selected from the group consisting of a primary cell, a non-embryonic

cell, a non-fetal cell, a differentiated cell, an undifferentiated cell, a somatic cell, an embryonic cell, a fetal cell, an embryonic stem cell, a primordial germ cell, a genital ridge cell, a cumulus cell, an amniotic cell, a fetal fibroblast cell, a uterine cell, an ovarian follicular cell, a cumulus cell, an hepatocyte, an embryonic germ cell, an adult cell, a cell isolated from an asynchronous population of cells, and a cell isolated from a synchronized population of cells where the synchronous population is not arrested in the G_0 stage of the cell cycle; (5) totipotent cells have a morphology of an embryonic germ cell.

The terms "precursor cell" or "precursor cells" as used herein refer to a cell or cells used to create a cell line of totipotent cells. Precursor cells can be isolated from any animal, preferably from a mammal, and more preferably from an ungulate. The precursor cell or cells may be isolated from nearly any cellular entity. For example, a precursor cell or cells may be isolated from blastocysts, embryos, fetuses, and cell lines (*e.g.*, cell lines established from embryonic cells), preferably isolated from fetuses and/or cell lines established from fetal cells, and more preferably isolated from *ex utero* animals and/or cell cultures and/or cell lines established from such *ex utero* animals. An *ex utero* animal may exist as a newborn animal, adolescent animal, yearling animal, and adult animal. The *ex utero* animals may be alive or post mortem. Examples of precursor cells include, but are not limited to, non-embryonic cells; non-fetal cells; differentiated cells; adult cells; somatic cells; embryonic cells; fetal cells; embryonic stem cells; primordial germ cells; genital ridge cells; uterine cells; amniotic cells; ovarian follicular cells; cumulus cells; cells isolated from an asynchronous population of cells; and cells isolated from a synchronized population of cells where the synchronous population is not arrested in the G_0 stage of the cell cycle; and any of the forgoing that are cultured, cultured as cell lines and/or totipotent.

The term "arises from" as used herein refers to the conversion of one or more cells into one or more cells having at least one differing characteristic. For example, (1) a non-totipotent precursor cell can be converted into a totipotent cell by utilizing features of the invention described hereafter; (2) a precursor cell can develop a cell morphology of an embryonic germ cell; (3) a precursor cell can give rise to a cultured cell; (4) a precursor cell can give rise to a cultured cell line; and (5) a precursor cell can give rise to a cultured permanent cell line. A conversion process can be referred to as a

reprogramming step. In addition, the term "arises from" refers to establishing totipotent embryos from totipotent cells of the invention by using a nuclear transfer process, as described hereafter.

The terms "reprogramming" or "reprogrammed" as used herein refer to materials and methods that can convert a non-totipotent cell into a totipotent cell. Distinguishing features between totipotent and non-totipotent cells are described previously. An example of materials and methods for converting non-totipotent cells into totipotent cells is to incubate precursor cells with a receptor ligand cocktail. Receptor ligand cocktails are described hereafter. In preferred embodiments, culturing of a cell is a sufficient stimulus to render a cell totipotent.

The term "reprogramming" or "reprogrammed" as used herein can also refer to materials and methods that can convert a cell into another cell having at least one differing characteristic. Also, such materials and methods may reprogram or convert a cell into another cell type that is not typically expressed during the life cycle of the former cell. For example, (1) a non-totipotent cell can be reprogrammed into an totipotent cell; (2) a precursor cell can be reprogrammed into a cell having a morphology of an EG cell; and (3) a precursor cell can be reprogrammed into a totipotent cell. An example of materials and methods for converting a precursor cell into a totipotent cell having EG cell morphology is described hereafter.

The term "isolated" as used herein in reference to cells refers to a cell that is mechanically separated from another group of cells. Examples of a group of cells are a developing cell mass, a cell culture, a cell line, and an animal. These examples are not meant to be limiting and the invention relates to any group of cells. Methods for isolating one or more cells from another group of cells are well known in the art. *See, e.g., Culture of Animal Cells: a manual of basic techniques* (3rd edition), 1994, R.I. Freshney (ed.), Wiley-Liss, Inc.; *Cells*: a laboratory manual (vol. 1), 1998, D.L. Spector, R.D. Goldman, L.A. Leinwand (eds.), Cold Spring Harbor Laboratory Press; and *Animal Cells: culture and media*, 1994, D.C. Darling, S.J. Morgan, John Wiley and Sons, Ltd.

The terms "cryopreservation" or "cryopreserved" as used herein refer to freezing a cell, embryo, or animal of the invention. The cells, embryos, or portions of animals of the invention are frozen at temperatures lower than 0°C, preferably lower

than -80°C, more preferably at temperatures lower than -140°C, and most preferably at temperatures lower than -196°C. Cells and embryos in the invention can be cryopreserved for an indefinite amount of time. It is known that biological materials can be cryopreserved for more than fifty years. For example, semen that is cryopreserved for more than fifty years can be utilized to artificially inseminate a female bovine animal. Methods and tools for cryopreservation are well-known to those skilled in the art. See, e.g., U.S. Patent No. 5,160,312, entitled "Cryopreservation Process for Direct Transfer of Embryos," issued to Voelkel on November 3, 1992, hereby incorporated by reference herein in its entirety, including all figures, tables, and drawings.

For the purposes of the present invention, the terms "embryo" or "embryonic" as used herein refer to a developing cell mass that has not implanted into the uterine membrane of a maternal host. Hence, the term "embryo" as used herein can refer to a fertilized oocyte, a cybrid (defined herein), a pre-blastocyst stage developing cell mass, and/or any other developing cell mass that is at a stage of development prior to implantation into the uterine membrane of a maternal host. Embryos of the invention may not display a genital ridge. Hence, an "embryonic cell" is isolated from and/or has arisen from an embryo.

An embryo can represent multiple stages of cell development. For example, a one cell embryo can be referred to as a zygote, a solid spherical mass of cells resulting from a cleaved embryo can be referred to as a morula, and an embryo having a blastocoel can be referred to as a blastocyst.

The terms "enucleated oocyte" or "enucleated recipient cell" as used herein refer to an oocyte which has had its nucleus removed. Typically, a needle can be placed into an oocyte and the nucleus can be aspirated into the inner space of the needle. The needle can be removed from the oocyte without rupturing the plasma membrane. This enucleation technique is well known to a person of ordinary skill in the art. *See,* U.S. Patent 4,994,384; U.S. Patent 5,057,420; and Willadsen, 1986, *Nature 320*:63-65. An enucleated oocyte can be prepared from a young or an aged oocyte. An enucleated oocyte is preferably prepared from an oocyte that has been matured, *in vitro* or *in vivo*, for some period of time. This time can vary, depending on the source species for the oocyte. For example, bovine oocytes are preferably matured for between 10 hours and

40 hours, more preferably for between 16 hours and 36 hours, and most preferably between 20 hours and 32 hours. In contrast, porcine oocytes are preferably matured for greater than 24 hours, and more preferably matured for greater than 36 hours. In particularly preferred embodiments, a porcine oocyte is matured for more than 40 hours, up to about 96 hours, more preferably from 42-54 hours, and even more preferably from 42 to 48 hours.

The terms "maturation" and "matured" as used herein refer to process in which an oocyte is incubated in a medium *in vitro*. Oocytes can be incubated with multiple media well known to a person of ordinary skill in the art. *See, e.g.*, Saito *et al.*, 1992, *Roux's Arch. Dev. Biol. 201*: 134-141 for bovine organisms and Wells *et al.*, 1997, *Biol. Repr. 57*: 385-393 for ovine organisms and also Mattioli *et al.*, 1989, *Theriogenology 31*: 1201-1207; Jolliff & Prather, 1997, *Biol. Reprod. 56*: 544-548; Funahashi & Day, 1993, *J. Reprod. Fert. 98*: 179-185; Nagashima *et al.*, 1997, *Mol. Reprod. Dev. 38*: 339-343; Abeydeera *et al.*, 1998, *Biol. Reprod. 58*: 213-218; Funahashi *et al.*, 1997, *Biol. Reprod. 57*: 49-53; and Sawai *et al.*, 1997, *Biol. Reprod. 57*: 1-6, each of which are incorporated herein by reference in their entireties including all figures, tables, and drawings. Maturation media can comprise multiple types of components, including microtubule inhibitors (*e.g.*, cytochalasin B), hormones and growth factors. Other examples of components that can be incorporated into maturation media are discussed in WO 97/07668, entitled "Unactivated Oocytes as Cytoplast Recipients for Nuclear Transfer," Campbell & Wilmut, published on March 6, 1997, hereby incorporated herein by reference in its entirety, including all figures, tables, and drawings. The time of maturation can be determined from the time that an oocyte is placed in a maturation medium to the time that the oocyte is subject to a manipulation (*e.g.*, enucleation, nuclear transfer, fusion, and/or activation).

Oocytes can be matured for any period of time: an oocyte can be matured for greater than 10 hours, greater than 20 hours, greater than 24 hours, greater than 36 hours, greater than 48 hours, greater than 60 hours, greater than 72 hours, and greater than 90 hours. The term "about" with respect to oocyte maturation refers to plus or minus 3 hours.

An oocyte can also be matured *in vivo*. Time of maturation may be the time that an oocyte receives an appropriate stimulus to resume meiosis to the time that the oocyte

is manipulated. Similar maturation periods described above for *in vitro* matured oocytes apply to *in vivo* matured oocytes.

Nuclear transfer may be accomplished by combining one nuclear donor and more than one enucleated oocyte. In addition, nuclear transfer may be accomplished by combining one nuclear donor, one or more enucleated oocytes, and the cytoplasm of one or more enucleated oocytes.

The term "young oocyte" as used herein refers to an oocyte that has been matured *in vitro* for a time less than or equal to the length of time between the onset of estrus and ovulation *in vivo*. For example, the onset of estrus is signaled by a surge in leutenizing hormone. A cow typically ovulates about 26 hours following the onset of estrus. Thus, a young oocyte is an oocyte matured for about 26 hours or less, preferably 16 to 17 hours. Methods for measuring the length of time between the onset of estrus and ovulation are well known to the skilled artisan. *See, e.g.*, P.T. Cupps, "Reproduction in Domestic Animals," Fourth Edition, Academic Press, San Diego, CA, USA, 1991. For horses, ovulation occurs about 33 hours after onset of estrus; for pigs, about 40 hours; for sheep and goats, about 24-36 hours; for dogs, about 40-50 hours; and for cats, about 24-36 hours. The term "young oocyte" may also refer to an oocyte that has been matured and ovulated *in vivo* and that is collected at about the time of ovulation. The term about in this context refers to +/- 1 hour.

Oocytes can be isolated from live animals using methods well known to a person of ordinary skill in the art. *See, e.g.*, Pieterse *et al.*, 1988, "Aspiration of bovine oocytes during transvaginal ultrasound scanning of the ovaries," *Theriogenology 30*: 751-762. Oocytes can be isolated from ovaries or oviducts of deceased or live born animals. Suitable media for *in vitro* culture of oocytes are well known to a person of ordinary skill in the art. *See, e.g.*, U.S. Patent No. 5,057,420, which is incorporated by reference herein.

Some young oocytes can be identified by the appearance of their ooplasm. Because certain cellular material (*e.g.*, lipids) have not yet dispersed within the ooplasm. Young oocytes can have a pycnotic appearance. A pycnotic appearance can be characterized as clumping of cytoplasmic material. For example, in bovines, a "pycnotic" appearance is to be contrasted with the appearance of oocytes that are older than 28 hours, which have a more homogenous appearing ooplasm.

The term "aged oocyte" as used herein refers to an oocyte that has been matured *in vitro* for a time greater than the length of time between the onset of estrus and ovulation *in vivo*. The term "aged oocyte" may also refer to an oocyte that has been matured and ovulated *in vivo* and that is collected later than about 1 hour after the time of ovulation. An aged oocyte can be identified by its characteristically homogenous ooplasm. This appearance is to be contrasted with the pycnotic appearance of young oocytes as described previously herein. The age of the oocyte can be defined by the time that has elapsed between the time that the oocyte is placed in a suitable maturation medium and the time that the oocyte is activated. The age of the oocyte can dramatically enhance the efficiency of nuclear transfer. For example, an aged oocyte can be more susceptible to activation stimuli than a young oocyte.

The term "ovulated in vivo" as used herein refers to an oocyte that is isolated from an animal a certain number of hours after the animal exhibits characteristics that it is in estrus. The characteristics of an animal in estrus are well known to a person of ordinary skill in the art, as described in references disclosed herein.

The terms "maternal recipient" and "recipient female" as used herein refer to a female animal which is implanted with an embryo for development of the embryo. A maternal recipient may be either homospecific or xenospecific to the implanted embryo. For example it has been shown in the art that bovine embryos can develop in the oviducts of sheep. Stice & Keefer, 1993, "Multiple generational bovine embryo cloning," *Biology of Reproduction 48*: 715-719. Implanting techniques are well known to a person of ordinary skill in the art. *See, e.g.*, Polge & Day, 1982, "Embryo transplantation and preservation," *Control of Pig Reproduction*, DJA Cole and GR Foxcroft, eds., London, UK, Butterworths, pp. 227-291; Gordon, 1997, "Embryo transfer and associated techniques in pigs," *Controlled reproduction in pigs* (Gordon, ed), CAB International, Wallingford UK, pp 164-182; and Kojima, 1998, "Embryo transfer," *Manual of pig embryo transfer procedures,* National Livestock Breeding Center, Japanese Society for Development of Swine Technology, pp 76-79, each of which is incorporated herein by reference in its entirety, including all figures, tables, and drawings.

The term "replication unit" as used herein refers to that portion of a chromosome or other DNA molecule capable of being replicated that is copied from a

given origin of replication. A chromosome in eukaryotes has many replication units. The term "origin of replication" refers to the location in a DNA molecule where its replication begins.

The term "essentially no homologous DNA" means that the DNA molecule in question comprises almost entirely heterologous DNA. Preferably, a molecule which contains essentially no homologous DNA comprises at least 98%, 99%, 99.5%, or 99.9% heterologous DNA when the number of base pairs of heterologous DNA in the molecule is divided by the overall number of base pairs in the molecule.

The term "homologous DNA" as used herein refers to DNA having the same nucleic acid sequence as DNA sequences present in cell nuclear DNA.

The term "germ line" refers to those cells which give rise to the reproductive cells of an organism. These cells contain the complete haploid genome of an organism and will pass these DNA molecules to the descendants of the organism in question.

The term "somatic cell" refers to those cells of an organism which are not involved in the production of gametes, e.g., they are not involved in passing the genome to the next generation of the organism in question.

Transgenic Embryos, Fetuses, and Animals

In yet another aspect, the instant invention relates in part to any embryos, fetuses, and animals emanating from totipotent mammalian cells of the invention, where one or more cells in these developing cell masses comprise at least one large heterologous nucleic acid construct, most preferably an artificial chromosome.

In preferred embodiments, at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90% of the cells of the embryos, fetuses, and animals emanating from totipotent mammalian cells of the invention comprise at least one large heterologous nucleic acid construct. Most preferably, between 90% and all of the cells of the embryos, fetuses, and animals emanating from totipotent mammalian cells of the invention comprise at least one large heterologous nucleic acid construct. Such embryos, fetuses, and animals are known in the art as being "transgenic." In certain embodiments, the large heterologous nucleic acid construct is an artificial chromosome, most preferably an ACEs or a euchromatin-based minichromosome.

The cells of the embryos, fetuses, and animals that comprise at least one artificial chromosome preferably comprise ten or fewer artificial chromosomes; more preferably comprise six or fewer artificial chromosomes, four or fewer artificial chromosomes, or two or fewer artificial chromosomes; and most preferably comprise one artificial chromosome. If the cells of the embryos, fetuses, and animals of the invention comprise more than one artificial chromosome, the artificial chromosomes may be identical or may differ from one another.

The term "transgenic" as used herein in reference to embryos, fetuses and animals refers to an embryo, fetus or animal comprising one or more cells that contain heterologous nucleic acids. In preferred embodiments, a transgenic embryo, fetus, or animal comprises one or more transgenic cells. While germ line transmission is not a requirement of transgenic embryos, fetuses, or animals as that term is used herein, in particularly preferred embodiments a transgenic embryo, fetus, or animal can pass its transgenic characteristic(s) through the germ line. In certain embodiments, a transgenic embryo, fetus or animal expresses one or more transgenes as transgenic RNA and protein molecules. Most preferably, a transgenic embryo, fetus or animal results from a nuclear transfer procedure using a transgenic nuclear donor cell.

Transgenic totipotent mammalian embryos can be established from cultured cybrids emanating from one or more nuclear transfer procedures, where one of the nuclear transfer procedures utilizes a totipotent mammalian cell harboring at least one artificial chromosome as a nuclear donor. A transgenic totipotent fetus can be established, for example, from a transgenic totipotent embryo that has been implanted into the uterus of a suitable female host. Cloned transgenic mammalian animals of the invention can be established from totipotent mammalian cells, totipotent mammalian embryos, and totipotent mammalian fetuses of the invention.

In certain embodiments, a transgenic animal embryo is produced by nuclear transfer of a nuclear donor cell into an enucleated recipient cell according to the following method: (a) a heterologous DNA molecule of greater than 100 kilobase pairs is introduced into one or more ungulate cells by microcell fusion; (b) the one or more cells are cultured to provide a cell culture; (c) a nuclear donor cell obtained from the cell culture is fused with an enucleated recipient cell to form a nuclear transfer embryo

comprising the heterologous DNA molecule; and (d) the nuclear transfer embryo is activated to provide the transgenic ungulate embryo.

In particularly preferred embodiments, method further comprises one or more of the following: the culturing step comprises selection for one or more markers of said heterologous DNA molecule, whereby at least 90% of cells in said cell culture comprise the heterologous DNA molecule; the transgenic animal is an ungulate selected from the group consisting of a bovine, an ovine, a caprine, and a porcine; the heterologous DNA molecule comprises one or more telomeres, one or more centromeres, and one or more origins of replication; the heterologous DNA molecule is contained within the cells of the transgenic ungulate embryo on a replication unit that comprises essentially no homologous DNA; the activated nuclear transfer embryo is cultured to at least the two cell stage, wherein at least 50% of the cells of the transgenic ungulate embryo comprise the heterologous DNA molecule; the nuclear donor cell is selected from the group consisting of a somatic cell, a primordial germ cell, an embryonic germ cell, and an embryonic stem cell; the heterologous DNA molecule comprises a plurality of copies of at least one transgene; the heterologous DNA molecule is between 100 kilobase pairs and 500 megabase pairs in size; and the heterologous DNA molecule is an artificial chromosome.

In yet another aspect, the invention features a method of using a cloned transgenic fetus or animal, where one or more cells of the fetus or animal comprise one or more large heterologous nucleic acid constructs. The method of using a cloned transgenic fetus or animal comprises the step of isolating at least one component from the fetus or animal.

The term "component" as used herein can relate to any portion of a fetus or animal. A component can be selected from the group consisting of fluid, biological fluid, cell, tissue, organ, gamete, embryo, and fetus.

The term "gamete" as used herein refers to any cell participating, directly or indirectly, in the reproductive system of an animal. Examples of gametes are spermatocytes, spermatogonia, oocytes, and oogonia. Gametes can be present in fluids, tissues, and organs collected from animals (*e.g.*, sperm is present in semen). For example, methods of collecting semen for the purposes of artificial insemination are well known to a person of ordinary skill in the art. *See, e.g., Physiology of*

Reproduction and Artificial Insemination of Cattle (2nd edition), Salisbury *et al.*, copyright 1961, 1978, WH Freeman & Co., San Francisco. However, the invention relates to the collection of any type of gamete from an animal.

The term "tissue" is defined previously. The term "organ" relates to any organ isolated from a fetus or animal, or any portion of an organ. Examples of organs and tissues are neuronal tissue, brain tissue, spleen, heart, lung, gallbladder, pancreas, testis, ovary, intestine, skin, and kidney. These examples are not limiting and the invention relates to any organ and any tissue isolated from a cloned animal of the invention.

In preferred embodiments, (1) fluids, biological fluids, cells, tissues, organs, gametes, embryos, and fetuses can be subject to manipulation; (2) the manipulation comprises isolating at least one component from an animal or fetus; (3) the manipulation comprises the step of cryopreserving the components; (4) the manipulation comprises the step of thawing components; (5) the manipulation comprises the step of separating the semen into X-chromosome bearing semen and Y-chromosome bearing semen; (6) the manipulation comprises methods of preparing the semen for artificial insemination; (7) the manipulation comprises the step of purification of desired polypeptide(s) from the component; (8) the manipulation comprises concentration of the components; and (9) the manipulation comprises the step of transferring one or more cloned cells, cloned tissues, cloned organs, and/or portions of cloned organs to a recipient organism (*e.g.*, the recipient organism may be of a different species than the donor source).

The term "separating" as used herein in reference to separating semen refers to methods well known to a person skilled in the art for fractionating a semen sample into sex-specific fractions. This type of separation can be accomplished by using flow cytometers that are commercially available. Methods of utilizing flow cytometers from separating sperm by genetic content are well known in the art. In addition, semen can be separated by its sex-associated characteristics by other methods well known to a person of ordinary skill in the art. *See*, U.S. Patents 5,439,362, 5,346,990, and 5,021,244, entitled "Sex-Associated Membrane Proteins and Methods for Increasing the Probability that Offspring Will Be of a Desired Sex," Spaulding, issued on August 8, 1995, September 13, 1994, and June 4, 1991 respectively, all of which are

incorporated herein by reference in their entireties including all figures, tables, and drawings.

Semen preparation methods are well known to someone of ordinary skill in the art. Examples of these preparative steps are described in *Physiology of Reproduction and Artificial Insemination of Cattle* (2nd. edition), Salisbury *et al.*, copyright 1961, 1978, W.H. Freeman & Co., San Francisco.

The term "purification" as used herein refers to increasing the specific activity of a particular polypeptide or polypeptides in a sample. Specific activity can be expressed as the ratio between the activity of the target polypeptide and the concentration of total polypeptide in the sample. Activity can be catalytic activity and/or binding activity, for example. Alternatively, specific activity can be expressed as the ratio between the concentration of the target polypeptide and the concentration of total polypeptide. Purification methods include dialysis, centrifugation, and column chromatography techniques, which are well-known procedures to a person of ordinary skill in the art. *See, e.g.*, Young *et al.*, 1997, "Production of biopharmaceutical proteins in the milk of transgenic dairy animals," *BioPharm 10(6)*: 34-38.

The term "transferring" as used herein can relate to shifting cells, tissues, organs, and/or portions of organs to an animal. The cells, tissues, organs, and/or portions of organs can be, for example, (a) developed *in vitro* and then transferred to an animal, (b) removed from an animal and transferred to another animal of a different specie, (c) removed from an animal and transferred to another animal of the same specie, (d) removed from one portion of an animal (*e.g.*, the leg of an animal) and then transferred to another portion of the same animal (*e.g.*, the brain of the animal), and/or (e) any combination of the foregoing. The term "transferring" can relate to adding cells, tissues, and/or organs to an animal and can also relate to removing cells, tissues, and/or organs from an animal and replacing them with cells, tissues, and/or organs from another source.

The term "transferring" as used herein can also refer to implanting one or more cells, tissues, organs, and/or portions of organs from the cloned mammalian animal into another organism. For example, neuronal tissue from a cloned mammalian organism can be grafted into an appropriate area in the human nervous system to treat neurological diseases such as Alzheimer's disease. Alternatively, cloned cells, tissues,

and/or organs originating from a porcine organism may be transferred to a human recipient. Surgical methods for accomplishing this preferred aspect of the invention are well known to a person of ordinary skill in the art. Transferring procedures may include the step of removing or deleting cells, tissues, or organs from a recipient organism before a transfer step.

Of particular interest are transgenic animals that express genes that confer resistance or reduce susceptibility to disease. Since multiple genes can be introduced on an ACEs, a series of genes encoding an antigen can be introduced, which upon expression will serve to immunize [in a manner similar to a multivalent vaccine] the host animal against the diseases for which exposure to the antigens provide immunity or some protection.

Also of interest are transgenic animals that serve as models of certain diseases and disorders for use in studying the disease and developing therapeutic treatments and cures thereof. Such animal models of disease express genes [typically carrying a disease-associated mutation], which are introduced into the animal on a MAC, preferably an ACEs, and which induce the disease or disorder in the animal. Similarly, MACs carrying genes encoding antisense RNA may be introduced into animal cells to generate conditional "knock-out" transgenic animals. In such animals, expression of the antisense RNA results in decreased or complete elimination of the products of genes corresponding to the antisense RNA. Of further interest are transgenic mammals that harbor MAC-carried genes encoding therapeutic proteins that are expressed in the animal's milk. Transgenic animals for use in xenotransplantation, which express MAC-carried genes that serve to humanize the animal's organs, are also of interest. Genes that might be used in humanizing animal organs include those encoding human surface antigens.

The invention relates in part to any disease or parasitic condition known in the art. *See, e.g., Hagan & Bruners Infectious Diesases of Domestic Animals* (7th edition), Gillespie & Timoney, copyright 1981, Cornell University Press, Ithaca NY. Examples of parasites include, but are not limited to, worms, insects, invertebrate, bacterial, viral, and eukaryotic parasites. These parasites can lead to diseased states that can be controlled by the materials and methods of the invention.

The term "regulatory element" as used herein refers to a DNA or RNA sequence that can increase or decrease the amount of product produced from another DNA or RNA sequence. The regulatory element can cause the constitutive production of the product (*e.g.*, the product can be expressed constantly). Alternatively, the regulatory element can enhance or diminish the production of a recombinant product in an inducible fashion (*e.g.*, the product can be expressed in response to a specific signal). The regulatory element can be controlled, for example, by nutrition, by light, or by adding a substance to the transgenic organism's system. Examples of regulatory elements well-known to those of ordinary skill in the art are promoters, enhancers, insulators, and repressors. *See, e.g., Transgenic Animals, Generation and Use*, 1997, Edited by L. M. Houdebine, Hardwood Academic Publishers, Australia, hereby incorporated herein by reference in its entirety including all figures, tables, and drawings.

The terms "promoters," "promoter," or "promoter elements" as used herein refer to a DNA sequence that is located adjacent to a DNA sequence that encodes a recombinant product. A promoter is preferably operatively linked to the adjacent DNA sequence. A promoter typically increases the amount of recombinant product expressed from a DNA sequence as compared to the amount of the expressed recombinant product when no promoter exists. A promoter from one organism can be utilized to enhance recombinant product expression from a DNA sequence that originates from another organism. In addition, one promoter element can increase an amount of recombinant products expressed for multiple DNA sequences attached in tandem. Hence, one promoter element can enhance the expression of one or more recombinant products. Multiple promoter elements are well-known to persons of ordinary skill in the art. Examples of promoter elements are described hereafter.

The terms "enhancers," "enhancer" or "enhancer elements" as used herein refer to a DNA sequence that is located adjacent to the DNA sequence that encodes a recombinant product. Enhancer elements are typically located upstream of a promoter element or can be located downstream of the coding DNA sequence (*e.g.*, the DNA sequence transcribed or translated into a recombinant product or products). Hence, an enhancer element can be located 100 base pairs, 200 base pairs, or 300 or more base pairs upstream of the DNA sequence that encodes the recombinant product. Enhancer

elements can increase the amount of recombinant product expressed from a DNA sequence above the increased expression afforded by a promoter element. Multiple enhancer elements are readily available to persons of ordinary skill in the art.

The terms "insulators," "insulator," or "insulator elements" as used herein refer to DNA sequences that flank the DNA sequence encoding the recombinant product. Insulator elements can direct the recombinant product expression to specific tissues in an organism. Multiple insulator elements are well known to persons of ordinary skill in the art. *See, e.g.*, Geyer, 1997, *Curr. Opin. Genet. Dev.* 7: 242-248, hereby incorporated herein by reference in its entirety, including all figures, tables, and drawings.

The terms "repressor" or "repressor element" as used herein refer to a DNA sequence located in proximity to the DNA sequence that encodes the recombinant product, where the repressor sequence can decrease the amount of recombinant product expressed from that DNA sequence. Repressor elements can be controlled by the binding of a specific molecule or specific molecules to the repressor element DNA sequence. These molecules can either activate or deactivate the repressor element. Multiple repressor elements are available to a person of ordinary skill in the art.

The terms "milk protein promoter," "urine protein promoter," "blood protein promoter," "tear duct protein promoter," "synovial protein promoter," "spermatogenesis protein promoter," and "mandibular gland protein promoter" refer to promoter elements that regulate the specific expression of proteins within the specified fluid or gland or cell type in an animal. For example, a milk protein promoter is a regulatory element that can control the expression of a protein that is expressed in the milk of an animal. Other promoters, such as β-casein promoter, melanocortin promoter, milk serum protein promoter, casein promoter, α-lactalbumin promoter, whey acid protein promoter, uroplakin promoter, and α-actin promoter, for example, are well known to a person of ordinary skill in the art.

The terms "insertion" and "introduction" as used herein in reference to artificial chromosomes or other large heterologous nucleic acid constructs refer to translocating one or more such artificial chromosomes or constructs from the outside of a cell to the inside of a cell. Insertion can be effected in at least two manners: by mechanical delivery and non-mechanical delivery.

The term "mechanical delivery" as used herein refers to processes that utilize an apparatus that directly or indirectly introduces DNA (*e.g.*, one or more artificial chromosomes) into one or more cells. Examples of mechanical delivery of DNA into cells include, but are not limited to, microinjection, particle bombardment, sonoporation, and electroporation.

The term "non-mechanical delivery" as used herein refers to non-mechanical processes such as diffusive processes, for example. For instance, non-mechanical delivery may be effected by introducing DNA (*e.g.*, an artificial chromosome) and one or more reagents to a medium bathing cell surfaces, where the reagents increase the probability that the DNA enters the cells. Such reagents are well known in the art, such as liposomes, acyl moieties, peptide moieties, saccharide moieties, and/or polyethylene glycol (PEG), for example. Such reagents may be complexed with the target molecule and the reagents may be introduced to cells *in vivo* and/or *ex vivo*. These examples are not meant to be limiting and the invention relates in part to any non-mechanical form of insertion.

The summary of the invention described above is not limiting and other features and advantages of the invention will be apparent from the following detailed description of the preferred embodiments, as well as from the claims.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The invention relates in part to totipotent cells comprising at least one artificial chromosome. These cells can be utilized in nuclear transfer processes for establishing cloned transgenic embryos, fetuses, and animals. These cells and other materials and methods of the invention represent an improvement towards establishing cloned transgenic animals.

One improvement towards establishing cloned transgenic animals is that large units of target DNA (*e.g.*, heterologous DNA) may be introduced to cells. Specifically, target DNA larger than 10 kb can be introduced into artificial chromosomes and these artificial chromosomes can be inserted into cells. Hence, genes and regulatory sequences larger than 10 kb in length can be inserted into one artificial chromosome and then introduced to cells; multiple types of regulatory sequences and multiple types

of genes can be inserted into an artificial chromosome and then introduced to cells; and multiple copies of a given target DNA sequence can be incorporated into an artificial chromosome and then incorporated into a cell. These are examples of improvements and the invention also relates to other improvements.

In another improvement, the invention provides materials and methods that can provide *in vitro* control of *in vivo* recombinant product expression levels. Specifically, artificial chromosomes comprising a known copy number of a target DNA can be selected before the artificial chromosome comprising the target DNA is inserted into a cell of interest. The number of copies can be quantified by a variety of techniques known in the art, such as Southern blot and FISH procedures, for example. Hence, in contrast to techniques currently applied in the art for establishing cloned transgenic animals, expression levels of one or more recombinant products can be controlled by the number of copies of target DNA present in an artificial chromosome that encode these recombinant products.

Another improvement towards establishing cloned transgenic animals is that the location of target DNA within a transgenic cell can be controlled. Artificial chromosomes remain distinct and separate from endogenous genomic DNA (*i.e.*, they exist as "extra-genomic" elements in the nucleus of the transgenic cell), thus providing a controlled and known locations of target DNA. This advantage is contrasted with many existing techniques for creating transgenic cells, which cause random insertion of target DNA into cell nuclear DNA. Because random insertion decreases efficiencies for establishing transgenic cells due to unproductive insertions that must be quantified *in vivo*, the materials and methods defined herein are improvements over the technology currently utilized in the art to clone transgenic animals.

The following descriptions define processes for establishing totipotent cells comprising one or more artificial chromosomes, introducing one or more artificial chromosomes into cells, and processes for utilizing totipotent cells that comprise one or more artificial chromosomes.

Establishing Totipotent Cells

The invention relates in part to establishing totipotent mammalian cells comprising one or more artificial chromosomes. These cells can be useful as nuclear

donors in nuclear transfer procedures. Utilizing these cells in nuclear transfer procedures can improve the efficiency of processes for establishing cloned and transgenic embryos, fetuses, and animals.

Totipotent mammalian cells can be established from nearly any type of precursor cell. Examples of precursor cells are non-embryonic cells; non-fetal cells; differentiated cells; somatic cells; embryonic cells; fetal cells; embryonic stem cells; primordial germ cells; genital ridge cells; cells isolated from an asynchronous population of cells; and cells isolated from a synchronized population of cells where the synchronous population is not arrested in the G_0 stage of the cell cycle; and any of the forgoing that are cultured, cultured as cell lines, immortalized, and/or totipotent. These examples are not meant to be limiting and the invention relates to any precursor cell known in the art.

Processes for establishing totipotent cells having at least one artificial chromosome comprise one or more of the following procedures: (1) inserting at least one artificial chromosome into a cell; (2) introducing a stimulus to a cell; (3) conducting one or more nuclear transfer procedures, which may optionally include fusion and/or activation steps; and (4) selecting cells that comprise at least one artificial chromosome. These procedures can be conducted in any order and each procedure may be repeated more than once.

Materials and methods for introducing at least one artificial chromosome into a cell and selecting for cells that comprise an artificial chromosome are defined hereafter. Materials and methods for (1) culturing cells; (2) passaging and plating cells; (3) preparing and administering a stimulus to cells; (4) preparing feeder cells; (5) and (6) establishing totipotent cells, are defined in PCT application number WO 98/39416 entitled "Method of Cloning Animals," Strelchenko *et al.*, filed March 5, 1998, which is hereby incorporated herein by reference in its entirety including all figures, tables, and drawings.

Preparing Artificial Chromosomes

Artificial chromosome expression systems (ACes) containing multiple copies of several transgenes were incorporated into bovine embryos produced using nuclear transfer technologies. In the first approach, ACes were incorporated into bovine

embryos by direct injection of ACes in enucleated oocytes just prior to their electrofusion with a nuclear donor cell to form a cybrid. Direct injection of ACes yield blastocysts in which up to 25% of the cells contained ACes.

In another approach, nuclear donor cells that contained ACes were enriched using hygromycin B and then used to generate nuclear transfer blastocysts that contain an artificial chromosome in 90% or more of cells for the purpose of producing cloned animals with specific traits or that express commercially relevant proteins in the mammary glands or other tissues.

The transfection of nuclear donor cells used in cloning has the distinct advantages of permitting the pre-selection of transgenic cells prior to nuclear transfer and providing for transgene incorporation in the majority of nucleated cells in the organism in the first generation. Because of the advantages of using transgenic nuclear donor cells, cloning has become the method of choice to generate transgenic animals. However, there are several limitations to current methods to produce transgenic nuclear donor cells. One limitation is that if the transgenes insert randomly into the genome, the expression of the transgene cannot be predicted. Position effects on transgene expression occur after all forms of gene delivery—microinjection, transfection, electroporation, infection, etc. In addition, random transgene integration is likely to cause mutations of critical genomic loci and generate a wide variety of abnormal phenotypes in cells and animals (Constantini et al., 1989; Rossant, 1991; Favor and Morawetz, 1992; Rijkers et al., 1994; Kurth, 1998; Woychik and Alagramam, 1998). If targeting to specific loci via homologous recombination is used, the size and copy number of the transgenes are limited. In addition, introducing several different transgenes into the same locus simultaneously would likely prove difficult.

An approach to overcome the limitations of both random transgene insertion and transgene targeting is to introduce into nuclear transfer embryos an entirely new chromosome, ACes. The advantage of ACes is that instead of genes being inserted at random into the existing chromosomes of a cell or embryo, genes are engineered onto a separate chromosome with its own structure for extra-genomic maintenance and replication. ACes with specific transgenes of interest have been transmitted through the germline of mice following their injection into the pronuclei of zygotes (Coet al. 2000). In addition, these artificial chromosomes have been transferred into cells *in vitro* via

microcell mediated chromosome transfer (Telenius et al. 1999) and other non-viral methods.

Artificial chromosomes are well known in the art. In particular, materials and methods for (1) preparing artificial chromosomes *de novo*, and (2) preparing recombinant vectors suitable for inserting heterologous DNA (*e.g.*, heterologous with respect to artificial chromosome DNA and/or heterologous with respect to cell nuclear DNA) into an artificial chromosome are well known in the art. . *See, e.g.*, Kereso *et al.*, 1996, *Chromosome Research 4*: 226-239, Holló *et al.*, 1996, *Chromosome Research 4*: 240-247, United States Patent No. 6,025,155, and United States Patent No. 6,077,697.

Artificial chromosomes can comprise multiple elements, including (1) one or more repressor elements; (2) one or more insulator elements; (3) one or more promoter elements; (4) one or more enhancer elements; (5) one or more units of target DNA; (6) one or more units of neutral DNA; (7) a centromere; (8) one or more origins of replication; and (9) one or more telomeres. These elements are defined previously and are well known in the art. These elements are examples and the invention relates in part to other DNA elements known in the art. These elements can be repeated in any number in an artificial chromosome, and can be located in any order in an artificial chromosome. Repeated elements can be contiguous and/or non-contiguous. Related but different elements can also exist in an artificial chromosome. For example, an artificial chromosome may comprise one or more copies of target DNA that encodes one type of recombinant product and one or more copies of another type of target DNA that encodes another type of recombinant product.

Target DNA can encode recombinant products including, but not limited to, ribozymes; antisense RNA; peptides; polypeptides; proteins; structural proteins; antibodies; enzymes; and portions, fragments, mutants, deletions, and fusions of any of the foregoing. Examples of recombinant products encoded by target DNA include hormones, enzymes, growth factors, clotting factors, apolipoproteins, receptors, drugs, pharmaceuticals, bioceuticals, nutraceuticals, oncogenes, tumor antigens, tumor suppressors, cytokines, viral antigens, parasitic antigens, bacterial antigens and chemically synthesized polymers and polymers biosynthesized and/or modified by chemical, cellular and/or enzymatic processes. Specific examples of recombinant products include proinsulin, insulin, growth hormone, androgen receptors, casein, milk

proteins, muscle proteins, insulin-like growth factor I, insulin-like growth factor II, insulin growth factor binding proteins, epidermal growth factor, TGF-α, TGF-β, platelet-derived growth factor (PDGF), angiogenesis factors (acidic fibroblast growth factor, basic fibroblast growth factor, and angiogenin), matrix proteins (Type I collagen, Type IV collagen, Type VII collagen, laminin), oncogenes (*ras*, *fos*, *myc*, *erb*, *src*, *sis*, *jun*), E6 or E7 transforming sequence, p53 protein, cytokine receptor, IL-1, IL-6, IL-8, IL-2, α, β, or γIFN, GMCSF, GCSF, viral capsid protein, and proteins from viral, bacterial and parasitic organisms. Other specific proteins or polypeptides which can be expressed include: phenylalanine hydroxylase, α-1-antitrypsin, cholesterol-7α-hydroxylase, truncated apolipoprotein B, lipoprotein lipase, apolipoprotein E, apolipoprotein A1, LDL receptor, scavenger receptor for oxidized lipoproteins, molecular variants of each, VEGF, and combinations thereof. Other examples are clotting factors, fibrinogen, factor VIII, Von Willebrands Factor, α-glucosidase, apolipoproteins, drugs, tumor antigens, viral antigens, parasitic antigens, monoclonal antibodies, and bacterial antigens. One skilled in the art readily appreciates that these proteins belong to a wide variety of classes of proteins, and that other proteins within these classes can also be used. These are only examples and are not meant to be limiting in any way.

It should also be noted that target DNA includes (1) nucleic acid sequences not normally found in the cells; (2) nucleic acid molecules which are normally found in the cells but not expressed at physiological significant levels; (3) nucleic acid sequences normally found in the cells and normally expressed at physiological desired levels; (4) other nucleic acid sequences which can be modified for expression in cells; and (5) any combination of the above.

Examples of promoter elements include, but are not limited to, milk protein promoter, urine protein promoter, blood protein promoter, tear duct protein promoter, synovial protein promoter, mandibular gland protein promoter, casein promoter, β-casein promoter, melanocortin promoter, milk serum protein promoter, α-lactalbumin promoter, whey acid protein promoter, uroplakin promoter, and α-actin promoter.

Materials and methods for manipulating DNA of mammalian cells are well-known to a person of ordinary skill in the art. *See, e.g.*, Molecular Cloning, a Laboratory Manual, 2nd Ed., 1989, Sambrook, Fritsch, and Maniatis, Cold Spring

Harbor Laboratory Press, hereby incorporated by reference in its entirety including all figures, tables, and drawings.

Introducing Artificial Chromosomes into Cells

Artificial chromosomes comprise DNA molecules that can be introduced into cells. Materials and methods for introducing DNA molecules into cells are well known in the art. The invention relates to introducing one or more artificial chromosomes into any type of cell. Examples of cell types are defined herein. Furthermore, artificial chromosomes can be introduced into cells when the cells are incorporated within fluids, tissues, organs, and animals.

DNA molecules can be introduced into cells by utilizing at least two types of processes. First, DNA can be inserted into cells by using mechanical processes, where DNA is physically inserted into a cell. Examples of mechanical processes are microinjection, sonoporation, electroporation, and particle bombardment. Second, DNA can be introduced into cells by using non-mechanical processes. Examples of diffusive processes known in the art are viral processes, non-viral processes, liposome-mediated cell fusion processes, peptide-mediated cell fusion processes, ligand/receptor processes, and diffusion processes that insert an entire complement of nuclear DNA into cells. The above-identified examples are not meant to be limiting and the invention relates to any combinations of materials and methods for introducing DNA into cells that are known in the art.

Materials and methods for DNA introduction processes are well known in the art. These methods include, but are not limited to, direct DNA transfer techniques, cell microinjection methods, micro particle bombardment processes, electroporation methods, cell fusion techniques, microcell fusion processes, lipid mediated transfer methods, lipofection processes, liposome mediated methods, protoplast regeneration processes, protoplast fusion methods, and polycation mediated techniques. *See, e.g.*, Hogan *et al.*, 1994, *Manipulating the Mouse Embryo, A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (see especially pages 255-264 and Appendix 3); Liszewski, 1998, "Non-viral Strategies for Gene Therapy," *Genetic Engineering News* (January 1, 1998): 13, 28, 32; Keown *et al.*, 1990, *Methods in Enzymology 185*: 527-537; Mansour *et al.*, 1988, *Nature 336*: 348-352; U.S. Patent No. 5,491,075; U.S. Patent No. 5,482,928; U.S. Patent No. 5,424,409; U.S.

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For a cell that is to be used as a nuclear donor, one or more artificial chromosomes can be introduced into the cell prior to placing the cell into the perivitelline space of the recipient oocyte, or after placing the cell into the perivitelline space and just prior to fusion. In other embodiments, one or more artificial chromosomes can be introduced into the recipient oocyte prior to fusion with the nuclear donor cell. In yet other embodiments, one or more artificial chromosomes can be introduced into a pre-formed cybrid prepared by fusion of a nuclear donor cell with a recipient oocyte.

Additionally, in certain preferred embodiments, one or more artificial chromosomes are not introduced into the cell, but rather allowed to associate with the exterior of either the nuclear donor cell or the recipient oocyte. In these embodiments, the artificial chromosome can be carried into the cybrid at the time of fusion.

Cells comprising one or more artificial chromosomes can be selected against cells that do not comprise an artificial chromosome by identifying the artificial chromosome directly and by detecting a marker present in an artificial chromosome.

Methods for identifying an artificial chromosome directly are well known in the art. Examples of such methods include FISH (fluorescent *in situ* hybridization) and chromosome karyotyping, where the artificial chromosome can be detected in one cell by simply counting the number of chromosomes in the cell. An artificial chromosome is present in a cell if the cell has one or more chromosomes in addition to the number of chromosomes normally present in such a cell. One cell that contains one or more artificial chromosomes can be utilized to establish a cell culture of such cells.

Markers are well known in the art. Examples of markers are drug resistance genes, such as genes that render a cell resistant to such drugs as neomycin, hygromycin, blasticidin S, and puromycin. These examples are not meant to be limiting. Other examples of markers are genes that express enzymes that directly or indirectly modify substrates, such as a gene which encodes β-galactosidase and a gene that encodes luciferase. If an artificial chromosome harbors more than one marker, multiple markers can be identified. Similarly, if a cell comprises more than one artificial chromosome, where each artificial chromosome harbors a distinct marker, multiple markers may be identified. Materials and methods for conducting such selection processes are well known in the art. Examples of processes for detecting markers in cells are polymerase chain reaction and FISH procedures by utilizing appropriate DNA probes. Hence, identifying cells comprising one or more artificial chromosomes can be accomplished by utilizing materials and methods well known in the art.

Processes that Utilize Totipotent Cells Comprising Artificial Chromosomes

The invention relates in part to processes that utilize totipotent cells comprising artificial chromosomes. These cells are preferably utilized as nuclear donors in nuclear transfer processes, where the nuclear donor is inserted into a recipient oocyte. Nuclear transfer procedures typically include a translocation step, where the nuclear donor is inserted into a recipient oocyte. Nuclear transfer procedures can optionally include a fusion step (*e.g.*, effected by one or more electric pulses and/or one or more fusion agents) and can optionally include an activation step (*e.g.*, electrostimulation and/or ionomycin coupled with DMAP). Nuclear transfer processes may include one or more nuclear transfer cycles and the various steps in each cycle can be executed in any order and may be repeated more than once in any cycle.

Nuclear transfer processes can give rise to cloned embryos, where the embryonic cells comprise one or more artificial chromosomes. One or more nuclear transfer cycles may be utilized to establish cloned embryos, fetuses, and animals of the invention. These cloned embryos can develop into a cloned fetus where the fetal cells comprise one or more artificial chromosomes. Cloned fetuses may be established, for example, when cloned transgenic embryos are implanted into an uterus of a suitable recipient female. Cloned transgenic animals may be established when cloned transgenic fetuses are allowed to develop into an animal.

Cells isolated from cloned embryos, fetuses, and animals can be subjected to selection processes defined previously to determine whether the cells comprise one or more artificial chromosomes. In addition, entire embryos and fetuses may be subjected to these selection processes.

Cells obtained from fetuses, embryos, and animals produced by the nuclear transfer procedures described herein can be used in a second nuclear transfer, or recloning, procedure. For example, blastomeres from a first nuclear transfer embryo can be used as nuclear donors, or used to establish a cell line, which cells are used as nuclear donors. Alternatively, a fetus can be harvested from a maternal host, and one or more cells used directly as nuclear donors, or used to establish a cell line, which cells are used as nuclear donors. Such cells can undergo selection for the presence of an artificial chromosome, as described herein. In addition, cells obtained from a transgenic animal can be used directly as nuclear donors, or used to establish a nuclear donor cell line.

Materials and methods for (1) conducting one or more nuclear transfer cycles; (2) conducting nuclear donor insertion processes; (3) conducting fusion processes; (4) conducting activation processes; (5) preparing oocytes as nuclear recipients; (6) preparing totipotent cells as nuclear donors; (7) culturing embryos; (8) manipulating embryos; (9) implanting one or more embryos into an uterus of an appropriate animal; (10) manipulating fetuses; and (11) using cloned animals, are defined in PCT application entitled "Method of Cloning Animals," Strelchenko *et al.*, filed March 5, 1998.

Examples

The examples below are non-limiting and are merely representative of various aspects and features of the present invention.

Example 1: Incorporating Artificial Chromosomes Into Cells

The present invention describes methods to incorporate artificial chromosomes into the nuclei of bovine embryos. One of these methods, using microcell mediated chromosome transfer into nuclear transfer donor cells, has yielded bovine blastocysts where greater than 90% of the cells contain one ACes/cell. Another method where ACes were injected into enucleated oocytes prior to fusion with a nuclear transfer donor cell has produced blastocysts where up to 25% of the cells contain one ACes/cell. The potential advantage of injecting ACes into enucleated oocytes is that transgenic embryos could be generated more quickly since the microcell fusion and selection processes would be avoided.

The combination of ACes and nuclear transfer technologies, as described herein, makes possible the generation of transgenic embryos containing one or more artificial chromosomes in a majority of cells. ACes are ideal chromosome vectors, they can be engineered with large payloads (Mb), isolated, delivered, and maintained as discrete non-integrating chomosomes for long-term stability in animals, and they eliminate the concerns of insertional mutagenesis. Another useful feature is that transgene expression from ACes can be evaluated in cell lines and mice prior to generating transgenic animals.

Generation of β-ACes.

The formation of the satellite DNA-based artificial chromosome has been described previously (Kereso, et al. 1996). The murine ACes described therein contained approximately 60 million base pairs (Mb) of DNA and included centromere, telomeres, blocks of murine satellite repeats, and two regions of heterologous DNA, including five copies of the lacZ gene encoding β-galactosidase and six copies of the hygromycin phosphotransferase gene which conferred hygromycin resistance ("β-ACes").

Generation of embryonic germ (EG) cells.

Genital ridge cells from bovine fetuses of age 50-60 days were isolated as follows. The genital ridges from a fetus were minced in 2 ml of TL-HEPES (Bio Whittaker #04-616F) containing 3 mg/ml protease (Sigma #p6911) using sterile surgical blades. The minced genital ridges were incubated at 37°C for 40-50 minutes, and then disaggregated by tituration with a 2 ml syringe and 25 gauge needle. The disaggregated genital ridge cell suspension was combined with 10 ml of TL-HEPES in a 15 ml sterile tube and centrifuged at 300 x g for 10 min. After aspiration of the supernatant, the disaggregated cells were resuspended in 10 ml of α-MEM medium (Gibco #32571-037) plus 10% FBS (Hyclone #A-111-D), and 0.1 mM β-mercaptoethanol. The resuspended cells were divided evenly between ten culture flasks (75 cm^2) containing mitotically inactivated fetal mouse feeder cells and culture with α-MEM medium (10 ml) supplemented with 25 ng/ml each of bovine basic fibroblast growth factor (bFGF) and human recombinant leukemia inhibitory factor (hrLIF).

After 7-10 days in culture, the culture flasks had become confluent with small, densely packed cells referred to as embryonic germ ("EG") cells. The EG cells were passaged into 25 culture dishes (5 x 10^6 cells /10 cm dish) containing 7 ml of α-MEM medium for the microcell fusion procedure.

Microcell Transfer of β-ACes to EG cells.

Microcells carrying β-ACes were produced from the rodent/hybrid cell line mM2C1 and transferred to bovine EG cells as described before (Telenius *et al.*, 1999) with minor modifications. 142 x 10^6 metaphase cells were loaded on five 50% Percoll (Pharmacia) cushions, generating 660 x 10^6 microcells. 540 x 10^6 microcells were fused to 1.25 x 10^8 recipient EG cells. Fusion was performed with 37% PEG 1450 (Sigma) for 3 minutes at 37°C, followed by drug-selection in 0.125 mg/ml hygromycin-B (Calbiochem) beginning 18-24 hours post-fusion. The duration of drug selection was 10-14 days. Hygromycin resistant cells were used for nuclear transfer 14-30 days following initiation of drug selection.

Example 2: Nuclear Transfer

Nuclear transfer of hygromycin-resistant EG cells was performed as described above (and in Strelchenko *et al.* 2000). Enucleated oocyte-donor cell complexes were fused by electroporation, activated chemically, and cultured for 7-10 days.

Isolation of ACes

β-ACes were isolated and purified by flow cytometry according to de Jong *et al.* (1999) except for the modification of the sheath buffer (10 mM Tris-HCl pH 7.5, 0.1 mM EDTA, 100 mM NaCl, 30 μM spermine, and 70 μM spermidine) (J-buffer). Just prior to use, the β-ACes were concentrated by centrifugation at 2500 x g for 15 min at 4°C in a swinging bucket rotor. All but 50 μl of the supernatant was removed and the pellet was resuspended by gently tapping the tube.

Microinjection of β-ACes into Cybrids Prior to Fusion

Enucleated oocytes with the donor cell in position under the *zona pellucida* (enucleated oocyte-donor cell complexes) were placed in a 25 μl drop of TL-HEPES medium on a 10 cm petri dish. 10-20 μl of concentrated β-ACes were placed in a separate drop of J-Buffer (25 μl). The drops were then covered with mineral oil.

A single ACes was drawn into a microinjection needle (Humagen # MIC-10,7 μm O.D.) and the needle was pressed against the oocyte plasma membrane adjacent to the nuclear donor cell. With controlled negative pressure, the membrane was drawn into the needle until the oocyte membrane ruptured. The cytoplasm drawn into the needle by this process was gently expelled back into the oocyte with the β-ACes such that the single β-ACes were placed adjacent to the nuclear donor cell.

The ACes-containing oocyte was then fused with the donor cell by electroporation using standard procedures (Strelchenko et al., 2000). The resulting embryos were activated and cultured for 7-10 days as described previously (Strelchenko et al., 2000).

Microinjection of β-ACes into Cybrids Subsequent to Fusion

After fusion of a nuclear donor cell with an enucleated oocyte, as described herein, the resulting cybrid is placed in a drop of TL-HEPES under mineral oil on a 10 cm petri dish. 10 to 20 μL of concentrated β-ACes are placed in a separate drop of J-Buffer. A single β-ACes is drawn into a microinjection needle (Humagen # MIC-10,7 μm O.D.) and the needle is pressed against the cybrid plasma membrane, preferably near the donor cell nucleus. With controlled negative pressure, the cybrid membrane is drawn into the needle until the oocyte membrane ruptures. The cytoplasm drawn into the pipet by this process is gently expelled back into the oocyte with the β-ACes such that the single β-ACes is placed adjacent to the donor cell nucleus.

Fluorescent in situ hybridization (FISH)

Metaphase preps of 7 to 10 day old expanded blastocysts were prepared by growing them for 12 hours in medium containing 1 μg/ml colchicine. The blastocyst was placed on a slide in 30 μl of 2:1 dH_2O:medium for 5 minutes. As much liquid as possible was removed and 30 μl of 0.01 M HCl/0.1% Tween-20 was added to the embryo until the *zona pellucida* dissolved and the cells started to disaggregate. A drop of cold fixative (3:1 methanol:acetic acid) was dropped onto the cells. The slide was allowed to dry and age at least 24 hours before proceeding with FISH. Extended embryo cultures were generated by culturing blastocysts in medium in 25 cm^2 culture flasks and were prepared for FISH in the same manner as the blastocysts with the exception that a portion of the attached cultures were dislodged from the tissue flask using an 18 gauge needle and a 1 ml pipet.

All general DNA manipulations were performed by standard procedures (*See, e.g.*, Molecular Cloning, a Laboratory Manual, 2nd Ed., 1989, Sambrook, Fritsch, and Maniatis, Cold Spring Harbor Laboratory Press). Genomic DNA was prepared from bovine EG cells using the Wizard genomic kit (Promega). Primers 5'-ATCCAGACAGACAAGACAAGACAT-3' and 5'-TTCCAGCGAGCGGCAAGGAC-3' were used to amplify a 1.9 Kb fragment from bovine satellite 1.709 (Accession No. X00979; Skowronski et al., 1984). PCR amplification conditions were as follows: 25 cycles at 90°C for 30 sec., 50°C for 30 sec., 72°C for 120 sec. The PCR product was purified and digested with EcoRI and

subcloned into the plasmid vector Bluescript SK⁻ (Stratagene), generating plasmid pBSAT-1.709. A biotinylated bovine satellite 1.709 DNA probe was prepared from pBSAT-1.709 DNA using the Biotin-Nick Translation Mix (Boehringer Mannheim). Digoxigenin labeled mouse major satellite DNA probe was prepared from plasmid pSAT-2 (Wong and Rattner, 1988) using the DIG-Nick Translation Mix (Boehringer Mannheim). FISH was performed as previously described (Pinkel et al., 1986). Bovine satellite probe does not cross hybridize to murine or hamster DNA sequences; the mouse major satellite probe does not cross hybridize with bovine DNA sequences.

Microcell Transfer

Two microcell transfer experiments resulted in successful generation of hygromycin-B resistant (hyg^R) EG cell colonies (>65). Hyg^R-EG cells were used as nuclear donor cells in nuclear transfer and 33 blastocysts were generated from 136 cybrids (24%). Expanded blastocysts (21) were placed in extended embryo culture to generate a cell line. Two of these embryo cell lines were proliferated to a point where FISH analysis could be performed. One embryo cell line comprised 1 β-ACes/cell in greater than 90% of the cells while the other cell line comprised 2 β-ACes/cell in greater than 90% of the cells.

The method of introducing β-ACes via microcell fusion has the advantage of producing blastocysts with little or no mosaicism. Since transgenic EG cells which have survived a similar selection regime as those described herein has produced live calves (Strelchenko et al., 2000), β-ACes-containing EG cells can be expected to be successful as nuclear donors for cloning bovines.

Injection of ACes into Enucleated Oocyte-Donor Cell Complexes

β-ACes were injected into 140 enucleated oocyte-donor cell complexes and the resulting enucleated oocyte-donor cell complexes were fused, activated and cultured as described elsewhere. β-ACes -injected cybrids developed at similar frequencies (39%) as the noninjected cybrids. Twenty-eight blastocysts were analyzed by FISH, of which 12 (43%) blastocysts contained β-ACes. Mosaicism in the transgenic blastocysts ranged from 1-25% (1 at 25%, 1 at 10%, 4 at 2%, and 6 at 1%).

While the invention has been described and exemplified in sufficient detail for those skilled in this art to make and use it, various alternatives, modifications, and improvements should be apparent without departing from the spirit and scope of the invention.

One skilled in the art readily appreciates that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The cell lines, embryos, animals, and processes and methods for producing them are representative of preferred embodiments, are exemplary, and are not intended as limitations on the scope of the invention. Modifications therein and other uses will occur to those skilled in the art. These modifications are encompassed within the spirit of the invention and are defined by the scope of the claims.

It will be readily apparent to a person skilled in the art that varying substitutions and modifications may be made to the invention disclosed herein without departing from the scope and spirit of the invention.

All patents, patent applications, and publications mentioned in the specification are indicative of the levels of those of ordinary skill in the art to which the invention pertains. All patents and publications are herein incorporated by reference to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference.

The invention illustratively described herein suitably may be practiced in the absence of any element or elements, limitation or limitations which is not specifically disclosed herein. Thus, for example, in each instance herein any of the terms "comprising", "consisting essentially of" and "consisting of" may be replaced with either of the other two terms. The terms and expressions which have been employed are used as terms of description and not of limitation, and there is no intention that in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed by preferred embodiments and optional features, modification and variation of the concepts herein disclosed may be resorted to by those skilled in the art, and that such modifications and

variations are considered to be within the scope of this invention as defined by the appended claims.

In addition, where features or aspects of the invention are described in terms of Markush groups, those skilled in the art will recognize that the invention is also thereby described in terms of any individual member or subgroup of members of the Markush group. For example, if X is described as selected from the group consisting of neomycin, hygromycin, and puromycin, claims for X being neomycin and claims for X being hygromycin and puromycin are fully described.

Other embodiments are set forth within the following claims.

CLAIMS

We claim:

1. A method of producing a transgenic ungulate embryo by nuclear transfer of a nuclear donor cell into an enucleated recipient cell, the method comprising:

(a) fusing a nuclear donor cell, or a nucleus thereof, with an enucleated recipient cell of the same species as the nuclear donor cell to form a nuclear transfer embryo, wherein a heterologous DNA molecule of greater than 100 kilobase pairs is injected into said oocyte prior to or following fusion, whereby said nuclear transfer embryo comprises said heterologous DNA molecule; and

(b) activating the nuclear transfer embryo to provide said transgenic ungulate embryo.

2. A method according to claim 1, wherein said transgenic ungulate embryo is cultured, and wherein said culturing step comprises selection for one or more markers of said heterologous DNA molecule.

3. A method according to claim 1, wherein the transgenic ungulate is selected from the group consisting of a bovine, an ovine, a caprine, and a porcine.

4. A method according to claim 1, wherein the heterologous DNA molecule comprises one or more telomeres, one or more centromeres, and one or more origins of replication.

5. A method according to claim 1, wherein the heterologous DNA molecule is contained within the cells of the transgenic ungulate embryo on a replication unit that comprises essentially no homologous DNA.

6. A method according to claim 1, wherein said nuclear transfer embryo is cultured to at least the two cell stage, wherein at least 50% of the cells of the transgenic ungulate embryo comprise the heterologous DNA molecule.

7. A method according to claim 1, wherein the nuclear donor cell is selected from the group consisting of a somatic cell, a primordial germ cell, an embryonic germ cell, and an embryonic stem cell.

8. A method according to claim 1, wherein the heterologous DNA comprises a plurality of copies of at least one transgene.

9. A method according to claim 1, wherein said heterologous DNA molecule is between 100 kilobase pairs and 500 megabase pairs.

10. A method according to claim 1, wherein said heterologous DNA molecule is an artificial chromosome.

11. A method of producing a transgenic ungulate from a transgenic ungulate embryo of claim 1, the method comprising:

transferring said transgenic ungulate embryo into a maternal host so as to produce a fetus of full fetal development and parturition to generate said transgenic ungulate.

12. A method according to claim 11, wherein prior to said transferring step, said transgenic ungulate embryo is cultured to at least the two cell stage.

13. A method according to claim 11, wherein at least 50% of the cells of the transgenic ungulate comprise the heterologous DNA molecule.

14. A method of producing a transgenic ungulate from a transgenic ungulate embryo of claim 1, the method comprising:

(a) transferring said transgenic ungulate embryo into a maternal host so as to produce a fetus;

(b) obtaining a nuclear donor cell from said fetus, wherein said cell comprises said heterologous DNA molecule;

(c) fusing said nuclear donor cell, or a nucleus thereof, with an enucleated recipient cell of the same species as the said cell to form a second nuclear transfer embryo, wherein said second nuclear transfer embryo comprises said heterologous DNA molecule;

(d) activating said second nuclear transfer embryo to provide a second transgenic ungulate embryo; and

(e) transferring said second transgenic ungulate embryo into a maternal host so as to produce a fetus of full fetal development and parturition to generate said transgenic ungulate.

15. A method according to claim 14, wherein prior to said transferring step, said transgenic ungulate embryo and/or said second transgenic ungulate embryo is cultured to at least the two cell stage.

16. A method according to claim 14, wherein at least 50% of the cells of the transgenic ungulate comprise the heterologous DNA molecule.

17. A method of producing a transgenic ungulate from a transgenic ungulate embryo of claim 1, the method comprising:

(a) transferring said transgenic ungulate embryo into a maternal host so as to produce a fetus;

(b) obtaining one or more cells from said fetus, wherein one or more of said cells comprise said heterologous DNA molecule, and culturing said one or more cells to obtain a cell culture;

(c) fusing a nuclear donor cell obtained from said cell culture, or a nucleus thereof, with an enucleated recipient cell of the same species as said nuclear donor cell to form a second nuclear transfer embryo, wherein said second nuclear transfer embryo comprises said heterologous DNA molecule;

(d) activating said second nuclear transfer embryo to provide a second transgenic ungulate embryo; and

(e) transferring said second transgenic ungulate embryo into a maternal host so as to produce a fetus of full fetal development and parturition to generate said transgenic ungulate.

18. A method according to claim 17, wherein prior to said transferring step, said transgenic ungulate embryo and/or said second transgenic ungulate embryo is cultured to at least the two cell stage.

19. A method according to claim 17, wherein at least 50% of the cells of the transgenic ungulate comprise the heterologous DNA molecule.

20. A method according to claim 17, wherein said culturing step comprises selection for one or more markers of said heterologous DNA molecule, whereby at least 90% of cells in said cell culture comprise said heterologous DNA molecule.

21. A transgenic ungulate embryo comprising one or more cells, wherein at least 50% of said one or more cells comprise an artificial chromosome.

22. A transgenic ungulate, wherein at least 50% of the cells making up said ungulate comprise an artificial chromosome.

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(54) Title: EMBRYONIC STEM CELLS AS NUCLEAR DONORS AND NUCLEAR TRANSFER TECHNIQUES TO PRODUCE CHIMERIC AND TRANSGENIC ANIMALS

(57) Abstract

The use of embryonic stem (ES) cells as donor nuclei for nuclear transfer (NT) procedures is provided. The method provides for the production of ES cell derived NT embryos. The use of ES cell derived NT embryos for producing chimeric non-human animals, preferably ungulates is also provided. Additionally, the use of transgenic ES cells as nuclear donors to provide transgenic embryos and transgenic offspring is taught.

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EMBRYONIC STEM CELLS AS NUCLEAR DONORS AND NUCLEAR TRANSFER TECHNIQUES TO PRODUCE CHIMERIC AND TRANSGENIC ANIMALS

Field of the Invention

In its broadest embodiment the present invention is directed to the use of embryonic stem (ES) cells as donor nuclei for nuclear transplantation of recipient oocytes.

The present invention is more particularly directed to a method of using ungulate embryonic stem (ES) cells as donor nuclei for nuclear transplantation (NT) of recipient ungulate oocytes.

The present invention is further directed to the use of the resultant ungulate ES cell derived NT embryos for the production of ungulate offspring.

The present invention is also directed to a novel method for producing chimeric ungulate embryos comprising introducing blastomeres obtained from a fertilized embryo into a NT embryo produced using an ES cell as the nuclear donor.

The present invention is further directed to the use of transgenic ES cells as donor nuclei for nuclear transplantation of recipient ungulate recipient oocytes.

The invention is also directed to the use of transgenic ES cell derived NT embryos for the production of transgenic ungulate offspring.

The invention is still further directed to a novel method for the production of transgenic chimeric embryos by combining blastomeres from a fertilized embryo with a transgenic ES cell derived NT embryo.

Background of the Invention

Embryo multiplication by nuclear transfer, also known as nuclear transplantation (NT), involves the transplantation of living nuclei from embryonic cells, or the whole embryonic cells themselves, into recipient cells, typically unfertilized eggs, followed by the fusion of the donor and recipient. Such transfers are typically made in order to increase the number of genetically identical embryos which can be obtained from elite genetic stock.

The earliest research relating to nuclear transfer of nuclei of embryonic cells into unfertilized eggs was performed in amphibians. Specifically, embryonic frog blastomere cells were separated and the nuclear material was reintroduced into frogs' eggs which had been enucleated. The results showed that adult offspring could be produced by nuclear transfer using blastula cells. See "Transplantation of Living Nuclei from Blastula Cells into Enucleated Frog Egg", Briggs, R., et al., Proc. Natl. Acad. Sci., 38, 455-463, 1972. Subsequent to this research, other experiments were performed in amphibians and amphibian eggs to determine if nuclear material from adult frog somatic or germinal cells could be transplanted into eggs and to ascertain whether the egg would develop into a normal larva. The results showed that adult offspring could not be produced by adult cells. See "Development and Chromosomal Constitution of Nuclear-

Transplants Derived from Male Germ Cells," Berradino, MA, et al., J. Exp. Zool., 176, 61-72, 1981.

Transplantation of mammalian living nuclear material into recipient oocytes has also been reported in the literature. The earliest successful reports of nuclear transplantation in higher mammals were achieved using sheep embryos wherein individual blastomeres from 8 and 16 cell embryos were used as the nuclear donors in enucleated or nucleated halves of unfertilized eggs. See "Nuclear Transplantation in Sheep Embryos," Willadsen, S.M. et al., Nature, 320, 64-65, 1986. About the same time, nuclear transplantation of bovine nuclear material was also reported in the literature in "Nuclear Transplantation in Bovine," Robl, J. et al., Theriogenology, 25, 1, 1986. However, the resultant embryos only developed for 43 days out of a normal nine month gestation period.

Fairly recently, substantial improvements in nuclear transplantation methods have been reported in the literature. Many of these improvements have been in the area of enhancing the number of viable embryos which may be obtained from a single fertilized embryo.

Essentially, once a fertilized embryo has reached a cleavage stage, which simply means that the embryo comprises at least two cells, it becomes feasible to transfer the nuclei from the cells, or the entire cells themselves, into recipient oocytes which have been enucleated, to thereby create multiple genetically identical embryos from such fusions. By allowing each of

the fused nuclear transfer embryos to develop to a multi-cell stage, and then repeating the nuclear transplantation procedure, it is possible to obtain a number of genetically identical nuclear transfer embryos from a single original donor embryo. Typically blastomeres isolated from pregastrulation embryos comprise the source of such donor nuclei.

An inherent limitation in the commercial use of nuclear transfer processes, as practiced to date, arises from the fact that there exist various inefficiencies in the nuclear transfer process. Most especially, not all of the nuclear fusions result in viable embryos. Also, not all of the embryos produced by nuclear fusion are capable of creating a viable (full term) pregnancy in a surrogate animal. This is evidenced, e.g., by the Robl et al. (Id.) reference discussed supra. Accordingly, substantial effort is currently being directed towards optimizing nuclear transfer techniques at each step of the process, so as to make the overall procedure more economically practical and reproducible.

Two patents which are illustrative of recent improvement in nuclear transplantation methods are U.S. Patent No. 4,994,384 to Prather et al. and U.S. Patent No. 5,057,042 to Massey.

Both of these patents relate to nuclear transplantation methods which enable the serial production of many genetically identical bovine animals. In these nuclear transplantation methods, oocytes are recovered

from the ovaries or reproductive tracts of cows. These oocytes are then selected for a particular stage of development, and are enucleated by physical aspiration through a transfer pipette, resulting in an enucleated oocyte which still retains its external membranes and which functions as a recipient donor nucleus or as a donor embryonic cell. Synchronously, a donor embryo at the proper stage of development, typically at the cleavage or morula stage, is manipulated so that one or more cells or blastomeres are removed from the embryo. The donor cell, which, of course, contains its nucleus, is then inserted into the perivitelline space of a recipient oocyte. An electronic pulse is then applied in order to fuse the membranes of the donor cell and the recipient oocyte, thus creating an activated, fused single cell embryo.

The resultant single cell nuclear transfer embryo is then cultured in vitro or in vivo in the oviduct of a mammal, until it reaches a stage of development wherein it may successfully be implanted into a recipient cow. This methodology results in a significant number of fused embryos which are viable, and which when transplanted surgically or non-surgically into the uteri of cows result in viable pregnancies and the production of genetically identical calves.

Since the basic nuclear transfer techniques were set forth in Prather et al. (Id.) and Massey (Id.), various aspects of nuclear transplantation methods have been modified or enhanced. For instance, much is now known

about synchronizing and optimizing the cell cycle stage of the donor and recipient cells prior to fusion.

For example, the timing of fusion has recently gained much attention by researchers as being an important variable in the rate of development of the resultant NT embryo to the blastocyst stage. This is significant since until the embryo attains the late morula or blastocyst stage it generally is not feasible to implant the NT embryo into a maternal recipient.

Most of the available evidence suggests that for optimal embryo cloning the oocyte should be activated prior to fusion. Similarly, studies conducted with frog NT embryos have shown that non-activated oocytes provide for the donor cell nuclei (erythrocytes) to be conditioned by the non-activated cytoplasm of the oocyte. However, the resultant NT embryos had to be processed through several serial transfers to maximize the rate of embryonic development. (Orr et al., Proc. Natl. Acad. Sci., USA, 83, 1369-1373, 1986). Moreover, the embryos never were able to attain the adult stage of development, but rather only grew to the tadpole stage. (Id.)

Another aspect of NT methodology which has been substantially improved comprises NT embryo culturing techniques. Culturing methods for fused nuclear transplant embryos have recently been substantially improved enabling the embryo to be cultured in vitro for longer periods of time prior to implantation into maternal recipients. See, e.g., Bondioli et al., "Bovine Nuclear

Transplantation," International Application No. PCT/US 88/01906, International Publication No. WO 88/09816, December 15, 1988; and Bondioli et al., Theriogenology, 33, 165-174, 1990 which teach development of bovine embryos to the morula or blastocyst stage in ligated sheet oviduct prior to transfer to maternal recipients, and Bondioli et al., 1990, (Id.) and Bondioli et al., "Bovine Embryo In Vitro Culture," International Publication No. WO 89/07135, (August 10, 1989); which relate to in vitro culture systems for nuclear transfer embryos. Also, cell-free embryo culture, which overcomes the 8-16 cell developmental block, has been reported in U.S. Patent No. 5,096,822 to Rosenkratz, Jr. et al.

Along similar lines, much effort has recently been directed toward maximizing the available supply of genetically identical donor nuclei (or nucleated donor cells) for use in nuclear transfer and for other genetic manipulations. Originally, donor nuclei were obtained directly from embryo cells. However, an inherent limitation to this strategy is that the maximum number of cells available from each embryo is small, on the order of 32-64 cells. It therefore has been the aim of researchers in this field to maximize the number of donor cells from each embryo. This would of course increase the likelihood of obtaining more genetically identical animals from a particular embryo.

Beyond a certain developmental state it is no longer possible to use every embryo cell for nuclear

transplantation because some of the cells are no longer totipotent. This occurs because some of the embryonic cells in the embryo have undergone significant cellular differentiation. By contrast, totipotent cells are cells which have not as yet significantly differentiated and thus may be used as donor nuclei or nucleated donor cells in nuclear transplantation. For example, it has been shown that cells of the inner cell mass (ICM), but not trophoblasts, of blastocyst stage bovine embryos (embryo of about 64 cells) may be used as donors for nuclear transfer.

Given the loss of totipotency which starts at about 64 cells into embryonic development, much effort has been directed toward producing continuous cultures of totipotent embryonic cell lines which, under proper growth conditions, remain both totipotent and undifferentiated until these cell lines are induced to differentiate. For example, such stem cell lines have been isolated from mice, and have putatively been obtained from bovine, ovine, hamster, mink and porcine blastocyst ICM cells. (See, e.g., Doetschman et al., "Establishment of hamster blastocyst-derived embryonic stem cells," Devel. Biol., 127, 224-227, 1988; Eistetter, "Pluripotent embryonal stem cells can be established from disaggregated mouse morulae", Devel. Growth and Diff. 31, 275-282, 1989; Flechon et al., "Characterization of ovine and porcine embryonic stem cells", J. Reprod. Ferti., 40, 25, 1990; Matsui et al., "Derivation of pluripotential embryonic

stem cells from murine primordial germ cells in culture," Cell, 70, 69, 891-897, 1992; Notarianni et al., "Maintenance and differentiation in culture of pluripotent embryonic cell lines from pig blastocysts," J. Reprod. Fert. Suppl., 43, 255-260, 1990; and Sukoyan et al., "Isolation and culturization of blastocyst-derived stem cell lines from American mink," Mol. Reprod. Devel., 33, 418-437, 1992.

See also, Weima et al., Proceedings 12th Int. Cong. in Animal Reprod., 2, 766-768, 1992, who disclose the isolation of purported permanent embryonic cell lines derived from bovine blastocysts. However, these cell lines were not tested for their ability to differentiate either in vitro or in vivo. Further, Saito et al., Roux's Arch. Dev. Biol., 201, 134-141, 1992, report the isolation of bovine "ES cell-like" cell lines obtained from bovine blastocyst stage embryos which remain viable after several passages.

However, despite the apparent availability of ES cell lines, to the best knowledge of the present inventors, there has not been reported in the literature the successful use of ES cells as nuclear donors for NT. Recently, Tsunoda et al., J. Reproduct. Fertil., 98, 537-540, 1993, reported attempts at utilizing mice ES cells as nuclear donors during NT. The authors disclosed that some of the resultant fusions developed to two-cell, four-cell, morulae and the blastocyst stage. However, upon implantation into maternal recipients, no live fetuses

were obtained. Moreover, the authors concluded based on these results that "[t]echniques for the development of reconstituted eggs with ES cells in vitro and for obtaining young following transfer to recipients should be improved."

Also, Saito et al., (Id.) disclosed the use of "ES-like cell lines" (which were cultured over several passages) as nuclear donors in NT techniques. However, the resultant embryos only reached the 8- to 16-cell stage.

The only reported successes with NT (fused embryo upon implantation results in viable pregnancy) have been obtained by nuclear transfer techniques using blastocyst-derived inner cell mass (ICM) and cultured ICM cells as nuclear donors. (See e.g., Sims et al., "Production of fetuses from totipotent cultured bovine inner cell mass cells"; Theriogenology, 34, 313, 1993). See also, Keefer et al., Theriogenology, 39, 342, 1993 who report the use of bovine inner cell mass (ICM) cells derived from bovine expanded blastocysts as donor nuclear for the production of nuclear transfer embryos. The authors further disclose that the resultant embryos when implanted into maternal recipients result in apparently successful pregnancies (100 and 190 gestation at reference date).

Further, Collas et al., Biol. Reprod. 45, 455-465, 1991, disclose the use of rabbit cleavage-, morula- and blastocyst- embryonic cells as nuclear donors for NT. Also, Smith et al., Biol. Reprod., 40, 1027-1035, 1989,

report the fusion of single cells derived from sheep 16-cell embryos, and the inner cell mass (ICM) of early blastocyst stage sheet embryos, with unfertilized enucleated secondary oocytes, the implantation of the resultant fused embryos into ewes, and the development of lambs which apparently express the phenotype of the nuclear donor breed.

However, while there are reported successes in the use of ICM cells as nuclear donors during NT for the production of NT embryos and offspring the use thereof is disadvantageous for at least several reasons. For example, in ungulates, the blastocyst inner cell mass (ICM) appears after the first round of differentiation which distinguishes the ICM cells from the cells of the trophectoderm. Moreover, cells of the trophectoderm, which constitute the majority of blastocyst cells, are not totipotent. Therefore, it is necessary when using ICM cells as nuclear donors to remove or otherwise separate the ICM cells from the trophectoderm. However, this has proven to be relatively difficult.

Further, and more importantly, the use of ICM cells as nuclear donors only results in a limited number of cells from any given embryo which are available for use as NT donor nuclei. As discussed supra, once the embryo has differentiated past a certain stage (generally around the blastocyst stage) the embryo contains a diminishing number of cells which are totipotent and which may be used as suitable NT donors. This of course greatly limits the

number of genetically identical offspring that can be produced from a single embryo.

Therefore, the use of ES cells as nuclear donors for NT would prove highly beneficial since it would both avoid the need to separate totipotent from differentiated cells prior to NT, and moreover would provide an essentially limitless supply of genetically identical cells for NT.

One method for the preparation of ungulate ES cells suitable as nuclear donors for NT is described in Example 6 herein below.

The discussion thus far has stressed the importance of embryonic stem cells for the production of cloned animals which are genetically identical. This, of course, is of great importance, especially in the agricultural industry for the production of identical animals having specific desirable traits, e.g., enhanced milk production or increased size. However, another important application of embryonic stem cell cultures is for the production of transgenic embryonic cell lines. Such transgenic embryonic stem cell lines may be used for <u>in vitro</u> research, or may be used as nuclear or cell donors for nuclear transfer to obtain transgenic embryos which, in turn, may be used for implantation into maternal recipients to produce transgenic animals.

Transgenic animals have tremendous research and commercial potential. For example, animals may be produced which contain disease causing genes and used as animal models for drug research. Also, transgenic animals

may be produced which contain genes providing for desirable traits, e.g., growth hormone genes.

The production of transgenic animals, and in particular transgenic ungulates, has been reported in the literature. For example, Simons et al., Bio/Technology, 6, 179-183, 1988, report the production of transgenic sheep by microinjection technique. Also, Rexroad et al., Mol. Reprod. Dev., 1, 164-169, 1989, report the integration of growth-regulating genes in sheep by microinjection of pronuclei.

Also, Biery et al., Theriogenology, 29(1), 224, 1988, disclose pronuclei injection of DNA into bovine zygotes; and McEvoy et al., Theriogenology, 33(4), 819-828, 1990, report the microinjection of very early stage embryos (one and two cell containing bovine ova) to create transgenic cattle. Further, Wilmut et al., Theriogenology, 33(1), 113-123, 1990, report gene transfer in order to modify milk production in bovines by the microinjection of bovine pronuclei with foreign DNA. The reference also discusses retroviral and stem cell transfer to blastocysts, and the use of sperm containing foreign DNA to fertilize eggs.

A significantly improved method to produce transgenic nonhuman animals, in particular bovines, entails microinjection of embryonic cells with desired genetic material, e.g., a homologous or heterologous DNA, and then use of the injected cells as nuclear donors for enucleated eggs. The injected cell and egg are fused, cultured to a requisite stage of development, and then implanted in a

maternal animal for the production of genetically transformed animals. This method has been reported to provide for much greater efficiencies than pronuclear injection. Hill et al., Theriogenology, 37, 222, 1992, and Bondioli et al., "Transgenic Animals", First, N. and Haseltine F. (eds.), Butterworth-Heinemann, Stoncham, MA., pp. 265-273, 1991.

See also a recent review article pertaining to the production of transgenic farm animals. Pursel et al., "Status of Research with Transgenic Farm Animals", J. Animal Sci., 71, 10-19, 1993.

Another important application of ES cells is for the production of chimeric animals. Mouse ES cells have been used extensively for the production of chimeras. (See, e.g., Beddington et al., Development, 105, 733-737, 1989); Bradley et al., Nature, 309, 255-256, 1984; Lu et al., Proc. Natl. Acad. Sci., USA, 77, 6012-6016, 1980, 104, 175-182, 1988). However, to date methods for obtaining chimeric animals have not utilized an initial NT procedure. Instead, chimeras are typically produced by injecting 5 to 20 ES cells into a fertilized embryo which is at the morula stage (Tokunage et al., Devel. Growth and Different, 34, 561-566, 1992) or the blastocyst stage (Bradley et al., Nature, 309, 255-256, 1989). A chimeric offspring is only produced when the ES cells themselves contribute to the ICM. Moreover, the efficiencies of such methods appear to vary from report to report.

It is has been hypothesized by some researchers that these differences may be attributable to factors such as the strain of mouse used to produce the ES cells, the methods used for handling the culture, and the age of the culture. Also, one study suggested that smaller ES cells gave a higher percentage of chimeras when they were injected into the blastocyst stage embryo (Brown et al., In Vitro Cell Dev. Biol., 28A, pp. 773-778, 1992). Additionally, methods for producing chimeric offspring in mice have been improved by combining tetraploid mouse embryos with mouse ES cells, resulting in offspring that are almost completely (about 95%) derived from ES cells (Nagy et al., Development, 110(3), 815-821, 1990). However, the resultant offspring died within 48 hours of birth.

Objects of the Invention

It is an object of the invention to provide a novel method of nuclear transplantation comprising introducing into a recipient oocyte a totipotent embryonic stem cell.

It is a more specific object of the invention to provide a novel method of nuclear transplantation comprising introducing into a recipient ungulate oocyte a totipotent ungulate embryonic stem cell.

It is an even more specific object of the invention to provide a novel method of nuclear transplantation

comprising introducing into a recipient bovine oocyte a totipotent bovine embryonic stem cell.

It is another object of the invention to provide a method for producing chimeric non-human animals, preferably ungulates, by introducing one or more blastomeres, preferably from a similar staged fertilized embryo, into nuclear transfer embryos produced using embryonic stem cells as the donor nuclei.

It is another object of the invention to provide a method for producing transgenic non-human animals, preferably ungulates, by using transgenic embryonic stem cells as donor nuclei during nuclear transfer methods.

It is a more specific object of the invention to provide a novel method of nuclear transfer comprising the following steps:

(i) obtaining a non-human embryonic stem cell culture;

(ii) disaggregating the embryonic stem cell culture into individual cells;

(iii) introducing an individual embryonic stem cell into an enucleated recipient oocyte;

(iv) fusing the embryonic stem cell and the enucleated recipient oocyte to produce a fused nuclear transfer embryo;

(v) activating the fused nuclear transfer embryo; and

(vi) culturing the activated fused nuclear transfer embryo under conditions which provide for the embryo to

attain a stage of development such that it may be implanted into a maternal recipient.

It is another specific object of the invention to provide a novel method for producing non-human chimeric embryos, preferably ungulate embryos, comprising the following steps:

(i) obtaining a non-human embryonic stem cell culture;

(ii) disaggregating the embryonic stem cell culture into individual cells;

(iii) introducing an individual disaggregated embryonic stem cell into an enucleated oocyte;

(iv) fusing the embryonic stem cell and enucleated oocyte to produce a fused nuclear transfer embryo;

(v) activating the fused nuclear transfer embryo;

(vi) culturing the fused nuclear transfer embryo;

(vii) introducing one or more blastomeres into the perivitelline space of the cultured nuclear transfer embryo; and

(viii) culturing the resultant chimeric embryo to a sufficient stage of development such that it may be successfully implanted into a maternal recipient and result in a viable pregnancy.

It is another object of the invention to provide a novel method of producing transgenic embryos comprising the following steps:

(i) obtaining a non-human embryonic stem cell culture;

(ii) disaggregating the embryonic stem cell culture into individual cells;

(iii) transfecting said individual cells with a desired polynucleotide and selecting for transgenic embryonic stem cells which comprise said polynucleotide stably integrated in their genome;

(iv) introducing a transgenic embryonic stem cell into an enucleated recipient oocyte;

(v) fusing the transgenic embryonic stem cell and enucleated recipient oocyte to produce a transgenic nuclear transfer embryo;

(vi) activating the transgenic nuclear transfer embryo; and

(vii) culturing the activated transgenic nuclear transfer embryo to a sufficient stage of development such that it may be implanted into a non-human maternal recipient and result in a viable pregnancy.

Detailed Description of the Invention

The present invention is generally directed to a method for using embryonic stem (ES) cells as nuclear donors in nuclear transplantation (NT) procedures. As discussed supra, to the best knowledge of the present inventors there has been no previous report of the successful use of embryonic stem cells to produce nuclear transplantation fetuses or offspring. Rather, previously disclosed NT methods have used embryonic cells as donor nuclei, derived from embryos which range in size from

about the cleavage stage to about the blastocyst stage. By contrast, the present invention provides for nuclear transfer embryos using embryonic stem (ES) cells as donor nuclei. This comprises a significant advance over previous nuclear transfer methods since embryonic stem (ES) cell lines may be obtained in essentially limitless numbers.

In general, nuclear transfer procedures comprise the transplantation of living nuclei from embryonic cells, or the whole embryonic cells themselves, into recipient cells, typically unfertilized eggs, followed by the fusion of the donor and recipient. By contrast, in the present invention, nuclear transfer will generally comprise the transplantation of a non-human embryonic stem cell into an enucleated oocyte, which cells are then fused to produce a nuclear transfer embryo.

Any non-human embryonic stem line which upon implantation into an enucleated oocyte and fusion results in a nuclear transfer embryo which provides for a viable pregnancy upon implantation into a maternal recipient is within the scope of the present invention. Preferably, however, ungulate embryonic stem cell cultures will be obtained and used as the donor nuclei. Methods for producing ungulate embryonic stem cell cultures, e.g., bovine and porcine embryonic stem cells have been described in the literature. See e.g., WO 90/06354; Saito et al., Roux's Arch. Dev. Biol., 201, 134-141, 1992; and

Weima et al., Proc. 12th Int. Cong. Animal Reprod., 2, 766-768, 1992.

In the preferred embodiment, the embryonic stem cells will be produced according to the method described in Example 6, infra.

In general, ES cell lines will be produced as follows:

(i) removing the zona pellucida from a preblastocyst stage embryo;

(ii) disaggregating the cells of the preblastocyst stage embryo into individual blastomeric cells; and

(iii) culturing the resultant blastomeres in a culture medium which prevents differentiation of the blastomeres but which permits the formation of embryonic stem cell colonies.

In the most preferred embodiment, the culturing step will be effected by co-culturing the blastomeres on a feeder cell layer which prevents differentiation, preferably fibroblasts or buffalo rat liver cells until embryonic stem cell monolayers are obtained. Generally, when using bovine pre-blastocyst of stage embryo cells as the embryonic stem cell progenitors this occurs about every 7 to 10 days, at which time the embryonic stem cell monolayer should be passaged into another cell culture medium, preferably a culture comprising another feeder cell layer which prevents differentiation of the blastomeres. At about this time the ES cells form a monolayer and start to look "embryoid" in appearance.

Although Example 6 illustrates the production of embryonic stem cells derived from pre-blastocyst stage embryonic stem cells, the present invention is not limited to the use of embryonic stem cells derived from pre-blastocyst stage embryonic cells, but should be recognized by those skilled in the art as embracing the use of any embryonic stem cells which are suitable for use in the subject NT method. However, preferably ES cell lines will comprise embryonic stem cell lines produced from blastomeres obtained from embryos ranging from about the cleavage stage to about the blastocyst stage. Therefore, for ungulates, embryonic stem cell lines will be produced from blastomeres obtained from ungulate embryos ranging in size from about 2 to about 64 cells.

To obtain embryonic stem cells useful for nuclear transfer it is necessary to disaggregate ES cell cultures to provide for single ES cells. This may be effected by chemical or mechanical means, however, mechanical means are generally preferred. In addition, a pronase treatment at about 0.1 to 1.0 mg/ml may also be effected to facilitate breaking down the outer layers of the ES cells.

In the preferred embodiment, embryonic stem cells which are to be used for nuclear transfer will be cut out of a embryonic stem cell monolayer comprised on a suitable feeder layer, preferably fibroblasts, and lifted off the feeder layer using a glass needle. The resultant mass of embryonic stem cells will then be incubated in a suitable disaggregation medium, e.g., a HEPES buffered medium

containing from about 0.1 to 1.0 mg/ml of pronase. The cells thus cultured will be under conditions which provide for disaggregation of the ES cells. Culturing for about one to four hours at 39°C in a pronase containing HEPES medium has been found to provide for suitable disaggregation. However, the time and disaggregation medium may be varied dependent upon, e.g., the age of the embryonic stem cell culture, and whether it is derived from pre-blastocyst or blastocyst embryonic cells, among other factors.

After the cells have been disaggregated into a single cell suspension they may be used in nuclear transfer procedures. Preferably the embryonic stem cells will be used within about 24 hours after disaggregation since the available data suggests that NT embryonic developmental rates decrease when the embryonic stem cells are used for nuclear transfer after this time.

The resultant embryonic stem cells, preferably ungulate stem cells, and most preferably bovine embryonic stem cells will then be introduced into suitable recipient enucleated oocytes. For the successful commercial use of techniques such as genetic engineering, nuclear transfer and cloning, oocytes must generally be matured <u>in vitro</u> before these cells may be used as recipient cells for nuclear transfer, and before they can be fertilized by the sperm cell to develop into an embryo. This process generally requires collecting immature (prophase I) oocytes from bovine ovaries obtained at a slaughterhouse

and maturing the oocytes in a maturation medium prior to fertilization or enucleation until the oocyte attains the metaphase II stage, which generally occurs about 18-24 hours post-aspiration. For purposes of the present invention, this period of time is known as the "maturation period." As used herein for calculation of time periods, "aspiration" refers to aspiration of the immature oocyte from ovarian follicles.

Additionally, metaphase I stage oocytes, which have been matured in vivo have been successfully used in nuclear transfer techniques. Essentially, mature metaphase II oocytes are collected surgically from either non-superovulated or superovulated cows or heifers 35 to 48 hours past the onset of estrus or past the injection of human chorionic gonadotropin (hCG) or similar hormone.

The stage of maturation of the oocyte at enucleation and nuclear transfer has been reported to be significant in the efficacy of NT methods. (See e.g., Prather, First and Differentiation, 48, 1-8, 1991). In general, successful mammalian embryo cloning practices use the metaphase II stage oocyte as the recipient oocyte because at this stage it is believed that the oocyte is sufficiently "activated" to treat the introduced nucleus as it does a fertilizing sperm. In domestic animals, and especially cattle, the oocyte activation period generally ranges from about 16-52 hours, preferably about 28-42 hours post-aspiration.

For example, immature oocytes may be washed in HEPES buffered hamster embryo culture medium (HECM) as described in Seshagine et al., Biol. Reprod., 40, 544-606, 1989 and then placed into drops of maturation medium consisting of 50 microliters of tissue culture medium (TCM) 199 containing 10% fetal calf serum which contains appropriate gonadotropins such as leutenizing hormone (LH) and follicle stimulating hormone (FSH), and estradiol under a layer of lightweight paraffin or silicon at 39°C.

After a fixed time maturation period, which ranges from about 10 to 40 hours, and preferably about 16 hours, the oocytes will be enucleated. However, prior to enucleation the oocytes will preferably be removed and placed in HECM containing 1 milligram per milliliter of hyaluronidase prior to removal of cumulus cells. This may be effected by repeated pipetting through very fine bore pipettes or by vortexing briefly. The stripped oocytes are then screened for polar bodies, and the selected metaphase II oocytes, as determined by the presence of polar bodies, are then used for nuclear transfer. Enucleation follows.

Enucleation may be effected by known methods, such as described in U.S. Patent No. 4,994,384. Essentially, metaphase II oocytes are either placed in HECM, optionally containing 7.5 micrograms per milliliter cytochalasin B, for immediate enucleation, or may be placed in a suitable medium, for example CR1aa, plus 10% estrus cow serum, and

then enucleated later, preferably not more than 24 hours later, and more preferably 16-18 hours later.

Enucleation may be accomplished microsurgically using a micropipette to remove the polar body and the adjacent cytoplasm. The oocytes may then be screened to identify those of which have been successfully enucleated. This screening may be effected by staining the oocytes with 1 microgram per milliliter 33342 Hoecht dye in HECM, and then viewing the oocytes under ultraviolet irradiation for less than 10 seconds. The oocytes that have been successfully enucleated can then be placed in a suitable culture medium, e.g., CR1aa plus 10% serum.

In the present invention, the recipient oocytes will preferably be enucleated at a time ranging from about 10 hours to about 40 hours after the initiation of <u>in vitro</u> maturation, more preferably from about 16 hours to about 24 hours after initiation of <u>in vitro</u> maturation, and most preferably about 16 hours after initiation of <u>in vitro</u> maturation.

A single embryonic stem cell will then be transferred into the perivitelline space of the enucleated oocyte. The embryonic stem cell and enucleated oocyte will then preferably be fused by methods which are known in the art. For example, the cells may be fused by electrofusion. Electrofusion is accomplished by providing a pulse of electricity that is sufficient to cause a transient breakdown of the plasma membrane. This breakdown of the plasma membrane is very short because the membrane reforms

rapidly. Essentially, if two adjacent membranes are induced to breakdown and upon reformation the lipid bilayers intermingle, small channels will open between the two cells. Due to the thermodynamic instability of such a small opening, it enlarges until the two cells become one. See U.S. Patent 4,997,384 by Prather et al., for a further discussion of this process. A variety of electrofusion media can be used including e.g., sucrose, mannitol, sorbitol and phosphate buffered solution. Fusion can also be accomplished using Sendai virus as a fusogenic agent (Graham, Wister Inot. Symp. Monogr. 9, 19, 1969).

Preferably, the ES cell and oocyte are electrofused in a 500 μm chamber by application of an electrical pulse of 90V for 15 μsec.

After fusion, the resultant embryonic stem cell nuclear transfer embryo will then be "activated" by placing the fused embryo in an activation medium. Table 1, infra, illustrates that the oocyte has to be activated prior to fusion in order to obtain a high developmental rate when using non-synchronized blastomeres obtained from embryos as nuclear transfer donors. In particular, it was found that developmental rates to morula and blastocyst stage for blastomere derived NT embryos improved significantly when fusion was induced after, rather than before, the time of activation.

Surprisingly, the present inventors have discovered that the reverse is true for ES cell derived NT embryos. Instead, developmental rates for ES cell NT embryos are

highest when fusion occurs prior to activation. This was demonstrated by varying the time of fusion from about 14 hours prior to activation to about 9 hours after activation as illustrated in Example 2, infra. This is a fundamental difference between embryo and ES cell NT procedures.

The fused NT embryos may be activated by a variety of methods. Such methods include, e.g., culturing the NT embryo at sub-physiological temperature, in essence by applying a cold, or actually cool temperature shock to the NT embryo. This may be most conveniently done by culturing the NT embryo at room temperature, which is cold relative to the physiological temperature conditions to which embryos are normally exposed.

Alternatively, activation may be achieved by known activation agents. For example, penetration of oocytes by sperm during fertilization has been shown to activate prefusion oocytes to yield greater numbers of viable pregnancies and multiple genetically identical calves after nuclear transfer. Also, treatments such as electrical and chemical shock may be used to activate NT embryos after fusion.

Additionally, activation may be effected by:

(i) increasing levels of divalent cations in the oocyte, and

(ii) reducing phosphorylation of cellular proteins in the oocyte.

This will generally be effected by introducing divalent cations into the oocyte cytoplasm, e.g., magnesium, strontium, barium or calcium, e.g., in the form of an ionophore. Other methods of increasing divalent cation levels include the use of electric shock, treatment with ethanol and treatment with caged chelators.

Phosphorylation may be reduced by known methods, e.g., by the addition of kinase inhibitors, e.g., serine-threonine kinase inhibitors, such as 6-dimethylamino-purine, staurosporine, 2-aminopurine, and sphingosine.

Alternatively, phosphorylation of cellular proteins may be inhibited by introduction of a phosphatase into the oocyte, e.g., phosphatase 2A and phosphatase 2B.

In the preferred embodiment, NT activation will be effected by briefly exposing the fused ES derived NT embryo to a HECM containing medium containing 5μM ionomycin and 1 mg/ml BSA, followed by a 5 minute dilution into HECM containing 30 mg/ml BSA within about 24 hours after fusion, and preferably about 4 to 9 hours after fusion.

The activated NT embryos may then be cultured in a suitable <u>in vitro</u> culture medium. Culture media suitable for culturing and maturation of embryos are well known in the art. Examples of known media, which may be used for bovine embryo culture and maintenance, include Ham's F-10 + 10% fetal calf serum (FCS), Tissue Culture Medium-199 (TCM-199) + 10% fetal calf serum, Tyrodes-Albumin-Lactate-Pyruvate (TALP), Dulbecco's Phosphate Buffered Saline

(PBS), Eagle's and Whitten's media. One of the most common media used for the collection and maturation of oocytes is TCM-199, and 1 to 20% serum supplement including fetal calf serum, newborn serum, estrual cow serum, lamb serum or steer serum. A preferred maintenance medium includes TCM-199 with Earl salts, 10% fetal calf serum, 0.2 MM Ma pyruvate and 50 μg/ml gentamicin sulphate. Any of the above may also involve co-culture with a variety of cell types such as granulosa cells, oviduct cells, BRL cells and uterine cells.

Another maintenance medium is described in U.S. Patent 5,096,822 to Rosenkrans, Jr. et al.. This embryo medium, named CR1, contains the nutritional substances necessary to support an embryo.

CR1 contains hemicalcium L-lactate in amounts ranging from 1.0 mM to 10 mM, preferably 1.0 mM to 5.0 mM. Hemicalcium L-lactate is L-lactate with a hemicalcium salt incorporated thereon. Hemicalcium L-lactate is significant in that a single component satisfies two major requirements in the culture medium: (i) the calcium requirement necessary for compaction and cytoskeleton arrangement; and (ii) the lactate requirement necessary for metabolism and electron transport. Hemicalcium L-lactate also serves as valuable mineral and energy source for the medium necessary for viability of the embryos.

Advantageously, CR1 medium does not contain serum, such as fetal calf serum, and does not require the use of a co-culture of animal cells or other biological media,

i.e., media comprising animal cells such as oviductal cells. Biological media can sometimes be disadvantageous in that they may contain micro-organisms or trace factors which may be harmful to the embryos and which are difficult to detect, characterize and eliminate.

Examples of the main components in CR1 medium include hemicalcium L-lactate, sodium chloride, potassium chloride, sodium bicarbonate and a minor amount of fatty-acid free bovine serum albumin (Sigma A-6003). Additionally, a defined quantity of essential and non-essential amino acids may be added to the medium. CR1 with amino acids is known by the abbreviation "CR1aa."

CR1 medium preferably contains the following components in the following quantities:

sodium chloride	- 114.7 mM
potassium chloride	- 3.1 mM
sodium bicarbonate	- 26.2 mM
hemicalcium L-lactate	- 5 mM
fatty-acid free BSA	- 3 mg/ml

In the preferred embodiment, after activation the NT embryos will be placed in CR1aa medium containing 1.9 mM DMAP for 4 hours followed by a wash in HECM and then cultured in CR1aa containing BSA.

After about three to four days the embryos may then be switched to CR1aa containing 10% fetal calf serum and cultured therein for the balance of their <u>in vitro</u> culture period. The resultant embryos may be nonsurgically transferred into recipient females anywhere from 5 to 9

days after the time of activation. However, this is only exemplary. Variation of the culture medium and culturing methods is within the level of skill in the art.

An alternative utility of the above-described ES derived NT embryos is for the production of chimeric embryos. As discussed previously, methods of using ES cells to produce chimeric embryos are generally known. However, methods to date have not combined these techniques with NT. The present inventors have developed a novel method of producing chimeric embryos whereby ES derived NT embryos are used as the embryonic recipients to which are introduced blastomeres from a embryo, preferably a similar staged fertilized embryo.

Essentially, this method will comprise the following steps:

(i) producing an ES cell derived NT embryo as described supra;

(ii) artificially activating the NT embryo;

(iii) culturing the cultivated NT embryo to a desired cell stage;

(iv) introducing one or more blastomeres from an embryo into the perivitelline space of the NT embryo and

(v) culturing the resultant chimeric embryo until the late morula or blastocyst stage of development so that it may be implanted into a maternal recipient.

Preferably the activation step (ii) will be effected as described previously, i.e. by a 4 minute exposure to 5μM ionomycin in HECM medium containing 1 mg/ml BSA

followed by a 5 minute dilution into HECM containing 30 mg/ml BSA.

The culturing step (iii) will be effected by known methods for culturing NT embryos and may be effected in vitro or in vivo. Preferably, culturing will be effected in vitro in CR1aa medium containing 1.0 mM DMAP for 4 hours followed by a wash in HECM, which is in turn followed by culturing in CR1aa containing BSA until the embryo reaches the desired cell stage. Preferably, this cell stage will range from about the cleavage stage to about the blastocyst cell stage which ranges from about 2 cells to about 64 cells into embryonic development. Most preferably, the NT embryos will be cultured until they develop into about 8 and 16-cell embryos.

Step (iv) will comprise the introduction of one or more blastomeres into the perivitelline space of the NT embryo which has been cultured to the desired cell stage. Preferably, about two blastomeres from a similar staged fertilized embryo will be placed in the perivitelline space of the NT embryo. However, the number of blastomeres may vary from one to about ten cells. The blastomeres which are transferred into the ES cell derived NT embryos may comprise blastomeres obtained from embryos ranging in size from about the cleavage stage to about the blastocyst stage.

In the preferred embodiment the blastomeres which are introduced will comprise tetraploid cells since it has been previously shown in the mouse that tetraploid cells

when used to produce chimeras preferentially contribute to the fetal placenta (Nagy et al., Development, 110(3), 815-821, 1990). Tetraploid embryos may be obtained by electrolyzing two-cell stage embryos resulting in one-cell stage embryos. Experiments conducted by the present inventors have demonstrated that when tetraploid cells are used as the introduced blastomeres it provides for around 50% of the resultant fetal tissue cells comprising cells derived from the ES cell clone.

After the chimeric embryo is produced it will then be cultured by known in vitro or in vivo methods prior to introduction into a maternal recipient where it will then develop into a viable chimeric offspring.

As discussed supra, the present invention further provides a novel method for producing transgenic embryos and offspring by nuclear transfer using transgenic embryonic stem cells as nuclear donors. In the preferred embodiment, transgenic ungulate animals, and most preferably transgenic bovines will be produced.

This will essentially comprise introduction of a desired polynucleotide into an embryonic stem cell, i.e., a particular DNA and/or RNA molecule, and the use of the resultant transgenic embryonic stem cell as a nuclear donor. Methods for the introduction of polynucleotide into cells in culture are well known in the art. Such methods include, e.g., electroporation, retroviral vector infection, particle acceleration, transfection, and microinjection.

The cells which contain the desired polynucleotide, e.g., a desired gene, which may be homologous or heterologous to the host cell, will then be selected according to known methods. For example, the cells may be co-transfected with a marker gene which provides for selection.

Once a culture of transgenic ES cells is obtained which contains a desired polynucleotide integrated into its genome, the individual cells may be used as NT donors in the above-described method. This is a particularly advantageous use of the present invention. Thereby, the transgenic ES cell will facilitate the production of many identical animals having an identical genetic modification, e.g., a desired gene integration. These animals will provide a means for in vivo study of the effects of the particular gene. Also, animals may be obtained which express a disease causing gene and used to assay potential drug therapies for treatment of the particular disease condition.

Moreover, transfection of ES cells in vitro with a desired polynucleotide will permit most of the genetic modification steps to be effected in vitro. This avoids the need to perform repeated breeding to introduce a desired gene into a particular genetic background. Also, one is able to characterize the donor cell genome in vitro (e.g., by use of suitable probes) that is not feasible when the donor is a primary embryo cell. Consequently, this procedure may be accomplished faster

than previous breeding methods. Also, since one has a high expectation that the offspring will express the desired gene, the need to screen animals is substantially reduced. Therefore, the invention should significantly reduce the cost of animal breeding, and the production of transgenic animals, particularly among larger domesticated animals, which have relatively prolonged gestation periods and which bear only one or a few offspring per pregnancy.

Additionally, genetically altered NT embryos obtained using genetically altered ES cells as the nuclear donor may themselves be used as a source of blastomeres which are then introduced into the perivitelline space of ES derived NT embryos for the production of transgenic chimeric embryos.

The present invention will now be further illustrated by reference to the following examples which are provided solely for purposes of illustration and are not intended to be in any way limitative.

Example 1

Essentially, the following example sets forth the preferred method for producing ES cell derived NT embryos. Oocytes are in vitro matured for about 16 hours followed by microsurgical enucleation by known methods such as are described supra. A single ES cell obtained by disaggregation of an ES cell culture is then transferred into the perivitelline space of the enucleated oocyte.

The ES cell is then fused with the enucleated oocyte by electrofusion in a 500 μm chamber by application of an electrical pulse of 90 v for 15 μsec. After fusion, the resultant ES derived NT embryo is then activated by a 4 minute exposure to 5 μM ionomycin in HECM medium containing 30 mg/ml BSA. The embryos are then cultured in CR1aa medium containing 1.0 mM DMAP for 4 hours followed by washing in HECM followed by culturing in CR1aa containing BSA.

About three to four days after culturing the embryos are then switched to CR1aa containing 10% fetal calf serum for the remainder of the in vitro culture period. The embryos are then nonsurgically transferred into recipient females at about 5 to 9 days after activation.

Example 2

In this example, the time in which the ES cell was fused into the enucleated oocyte was altered. The time of activation remained constant at 24 hrs after the initiation of oocyte maturation. The time of fusion ranged from 14 hrs prior to activation to 9 hrs after the time of activation. For purposes of comparison, at a number of these time points, 32-cell stage blastomeres were also used as donor nuclei. Blastomeres were isolated and transferred into recipient oocytes by the methods described supra.

Table 1 shows that developmental rates to morula and blastocyst stages differed depending on the type of donor

nuclei used and the time of fusion. Developmental rates to morula and blastocyst stage for blastomere derived NT embryos were improved when fusion was induced after the time of activation; whereas, developmental rates for ES cell NT embryos were highest when fusion was prior to activation. As discussed supra, this is a fundamental difference between embryo and ES cell NT procedures.

TABLE 1. Effect of time of fusion with respect to activation on the developmental rate to morula and blastocyst stages

Time of Fusion*

Donor Nuclei Source	-14	-9	-7	-4	-1	+1	+5	+9
Blastomere				10/74 0%		7/105 7%	62/475 13%	13/80 16%
ES Cell	0/243 0%	59/602 10%	10% ‡	103/1045 10%	13/549 2%	16/526 3%	47/2195 2%	8/421 2%

* Number of hours fusion pulse was prior to or after the time of activation
(Example-1 = fusion pulse given 1 hour prior to activation)

‡ Averaged % developmental rate from two treatments within the same time point

A number of pregnancies were established with ES cell NT embryos, however, to date all of these have aborted prior to 60 days of pregnancy. Interestingly, the longest pregnancy was 35 days when the oocyte was activated prior

to fusion, while two pregnancies of 50 to 55 days were obtained when fusion was induced prior to activation. Thus, it appears that the ES cell was more easily or more completely remodeled when it was exposed to the oocyte's cytoplasm for a period of time prior to activation.

Example 3

In this example the time of fusion remained constant and the time of activation was altered. All of the ES cell NT embryos were fused at about 20 hours after the initiation of maturation. In the first group (AC24) the ES cell NT embryos were activated 4 hrs later at 24 hours after the initiation of maturation using the ionomycin and DMAP protocol described above. The second group (AC39) was not activated until 19 hours later using ionomycin and DMAP. This second group was held in CR1aa during the 19 hour waiting period. The results in table 2 shows that altering the time of activation as much as 19 hours after fusion does not significantly affect developmental rates to morula and blastocyst stage.

TABLE 2. The effect of time of fusion on developmental rates for ES cell NT embryos

Treatment	No. NTs To Culture	No. NTs to Mor and Blast	Developmental Rate
ES Cell fused 4 hrs before Act. (AC24)	1045	103	10%
ES Cell fused 19 hrs. before Act (AC39)	568	43	8%

Example 4

Isolated single cell suspension ES cells range from 15 μm to 21 μm in cell diameter. With this diversity in size, there could be a subpopulation of ES cells that have a greater chance of developing into an embryo. Therefore, NT developmental rates to morula and blastocyst stages were monitored for different size donor ES cells. In this experiment the time of fusion and activation remained constant. Fusion was induced at 20 hrs and activation at 24 hrs after the initiation of maturation. The results of this experiment are provided in table 3. These data show that the smaller ES cell group has higher developmental rates to morula and blastocyst stage than the two other groups of ES cells with larger cell diameter. The reasons for these results may be attributable to the genetic makeup and/or the cell stage of the donor nucleus.

TABLE 3. The effect of ES cell diameter (15 μm, 18 μm and 21 μm) on developmental rates of ES cell NT embryos

Treatment	No. NTs to Culture	No. NTs to Mor & Blast	Developmental Rate
ES15	593	117	20%
ES18	703	88	13%
ES21	642	67	10%

Example 5

As previously mentioned, an ES cell NT embryo when transferred into a recipient animal will implant and differentiate into a fetus. Several fetuses have been shown to have beating hearts when examined through ultrasound visualization. However, the heartbeat has always been lost before sixty days of gestation. The cause for this loss was unknown since there have been no gross anatomical abnormalities observed.

In order to overcome these losses, chimeric embryos were produced. This procedure entails the transfer of two blastomeres from an eight to 16-cell stage fertilized embryo into the perivitelline space of an eight to 16-cell stage ES cell clone. The chimeric embryo is allowed to develop to the blastocyst stage and then is transferred to a recipient animal.

There were two types of blastomeres transferred into the ES cell clones. One was the normal diploid blastomeres and the other being blastomeres from a presumably tetraploid embryo. Tetraploid embryos were produced by electrofusing two-cell stage embryos resulting

in one-cell stage embryos. The fused tetraploid embryos were then cultured in vitro until they reached the 8- to 16- cell stage at which time blastomeres from these embryos were used to produce chimeric embryos. Tetraploid cells were used because it has been shown in the mouse that when tetraploid cells were used to make chimeras these cells preferentially contribute to the placenta of the fetus (Nagy et al., 1992). When these cells were combined with entirely mouse ES cells, less than 5% of the cells in the resulting fetus and greater than 95% of the cells in the placenta were derived from the tetraploid cells.

The pregnancy results from these studies are presented in table 4. At this time five of the eleven on-going pregnancies have already passed the mid-point of gestation. Three of these pregnancies are presumably from tetraploid chimeras and two from diploid chimeras. Tissue samples from a tetraploid fetus that was taken on day 85 of gestation showed that around 50% of the cells in three different tissues were indeed derived from the ES cell clone.

TABLE 4. Pregnancy rates for ES cell clone chimeras

Treatment	No. Embryos Transferred	No. Pregnancies Initiated (%)	No. Pregnancies On-Going (>30 days)
Diploid Chimeras	109	13	7
Tetraploid Chimeras	82	14	4

Example 6

In this example a line of embryonic stem cells are produced. Preblastocyst stage bovine embryos were obtained from in vivo fertilizations and in vitro fertilizations. These embryos ranged in size from the 8-cell stage to the 64-cell stage, and were selected such that they were between 2 and 5 days from time of fertilization. These embryos were then treated with 1 mg/ml of pronase to remove the zona pellucida. Some of these embryos were then placed in phosphate buffered saline (PBS) medium containing 7.5 μg/ml of cytochalasin B to reduce the compactness of the embryo structure and therefore facilitate the disaggregation of cells. However, some embryos were disaggregated without a cytochalasin B pretreatment.

Individual blastomeres, removed from the preblastocyst stage embryos ranging in size from between 8 to 64 cells, were then placed either on top or underneath a mouse fibroblast feeder cell layer prepared according to the method described infra. In particular, the feeder cell layer was prepared from mouse fetuses at ten to twenty days of gestation. The head, liver, heart and alimentary tract were removed from the fetuses and the remaining tissue was washed and incubated at 37°C in 0.05% trypsin-0.02% EDTA. Loose cells were then cultured in tissue culture flasks containing MEM-α supplemented with penicillin, streptomycin, 10% fetal calf serum and 0.1 mM

ß mercaptoethanol. The feeder cell cultures were established over a two- to three-week culture period at 37°C, 5% CO_2 and at 100% humidity. These cells were then treated with mitomycin C at 10 µg/ml prior to their usage as feeder cells.

The mitomycin C-pretreated fibroblast layer was then used as a feeder cell layer for the blastomeres. In some experiments the individual blastomeres were placed on top of the feeder cell layer. However, ES cell lines were more readily established, and differentiation was better inhibited, when the blastomeres were placed beneath the feeder layer. It is believed that this enhanced cell-to-cell contact provides for the blastomeres to be more in contact with membrane associated differentiating inhibiting factors such as leukemia inhibiting factor (LIF). Cells placed on top of the feeder layer had lesser cell-to-cell contact between the blastomeres and feeder cells. Also, the blastomeres occasionally differentiated into trophoblast vesicles.

Every two to three days, the MEM-α plus 10% FCS growth medium was replaced. After the cells had been cultured for a total of about seven to ten days, embryonic stem cell monolayers were obtained. Also, around this time into the culturing, the blastomeres (which now had become ES cell lines) start to differentiate and exhibit an embryoid-like appearance.

Accordingly, the cells are passaged at this time onto new feeder layers. This was effected mechanically using a

fine glass needle to cut the ES cell monolayer into smaller cell clusters. These cell clusters were then repeatedly pipetted at a 1:100 dilution onto fresh fibroblast feeder layers.

This method has resulted in the generation of numerous ES cell lines from preblastocyst-derived embryos from the 8- to 64-cell stage, and has provided for both male and female ES cell lines.

The ES cell data obtained by the above-described method is presented in Table 5 below.

TABLE 5

EMBRYONIC STEM CELL DATA

Starting Embryo	No. Starting Embryos	No. ES Cell Lines	Efficiency
Blastocyst	42	22	53%
Morula	11	6	55%
8-16 Cell	12	8	67%
4-cell	30	0	0%

We claim:

1. A method of nuclear transfer comprising the following steps:

(i) obtaining a non-human embryonic stem cell culture;

(ii) disaggregating the embryonic stem cell culture into individual cells;

(iii) introducing an individual embryonic stem cell into an enucleated recipient oocyte;

(iv) fusing the embryonic stem cell and enucleated recipient oocyte to produce a nuclear transfer embryo;

(v) activating the nuclear transfer embryo; and

(vi) culturing the activated nuclear transfer embryo to a sufficient stage of development such that it may be implanted into a non-human maternal recipient.

2. The method of claim 1 wherein the non-human embryonic stem cell culture comprises an ungulate stem cell culture.

3. The method of claim 2 wherein the ungulate is selected from the group consisting of pigs, horses, cows, antelope, goats and sheep.

4. The method of claim 3 wherein the ungulate is a cow.

5. The method of claim 1 wherein enucleation is effected about 16 hours after the initiation of in vitro maturation.

6. The method of claim 1 wherein fusion is effected by electrofusion.

7. The method of claim 6 wherein electrofusion is effected by application of an electrical pulse of about 9V for about 15 μseconds.

8. The method of claim 1 whereby activation of the fused nuclear transfer embryo is effected by means of fertilization by sperm, cold culture at a temperature which are below physiological temperature, electric shock or chemical treatment.

9. The method of claim 1 wherein activation is effected by:

(i) increasing intracellular levels of divalent cations in the oocyte; and

(ii) reducing phosphorylation of cellular proteins in the oocyte.

10. The method of claim 9 wherein divalent cations are increased by the addition of a divalent cation to the oocyte cytoplasm.

11. The method of claim 10 wherein said divalent cation is selected from the group consisting of calcium, magnesium, strontium and barium.

12. The method of claim 10 wherein said divalent cation is a free calcium ion.

13. The method of claim 10 wherein said divalent cation is introduced in the form of an ionophore.

14. The method of claim 13 wherein said ionophore is selected from the group consisting of ionomycin and A23187.

15. The method of claim 9 wherein divalent cations are increased by electric shock, treatment with ethanol or treatment with a caged chelator.

16. The method of claim 9 wherein phosphorylation of cellular proteins is inhibited by the addition of a kinase inhibitor.

17. The method of claim 16 wherein said kinase inhibitor is a serine-threonine kinase inhibitor.

18. The method of claim 17 wherein said serine-threonine kinase inhibitor is selected from the group

consisting of 6-dimethyl-aminopurine, stauropurine, 2-aminopurine and sphingosine.

19. The method of claim 9 wherein phosphorylation of cellular proteins is inhibited by the addition of a phosphatase.

20. The method of claim 19 wherein said phosphatase is phosphatase 2A or phosphatase 2B.

21. The method of claim 1 whereby activation is effected by exposure to 5μM ionomycin in HECM medium containing 1 mg/ml BSA followed by dilution into HECM containing 30 mg/ml of BSA.

22. The method of claim 1 wherein the embryonic stem cells are disaggregated by incubation in a HEPES buffered medium containing pronase.

23. The method of claim 1 wherein the disaggregated embryonic stem cells are introduced into the enucleated oocyte less than about 24 hours after disaggregation.

24. The method of claim 1 wherein the activated fused nuclear transfer embryo is cultured in a CR1aa medium.

25. The method of claim 1 wherein the cultured activated nuclear transfer embryos are implanted into a maternal recipient from about 5 to 9 days after culturing.

26. The method of claim 1 wherein the embryonic stem cell introduced into the enucleated oocyte ranges in size from about 15 μm to 21 μm in cell diameter.

27. The method of claim 1 wherein activation is effected within about 24 hours after fusion.

28. A nuclear transfer embryo produced according to claim 1.

29. A nuclear transfer embryo produced according to claim 2.

30. A nuclear transfer embryo produced according to claim 3.

31. A nuclear transfer embryo produced according to claim 4.

32. A method for producing a chimeric non-human embryo comprising:

(i) obtaining an embryonic stem cell culture;

(ii) disaggregating said embryonic stem cell culture into individual cells;

(iii) introducing said disaggregated embryonic stem cell into an enucleated oocyte;

(iv) fusing the embryonic stem cell and enucleated oocyte to produce a nuclear transfer embryo;

(v) activating the nuclear transfer embryo;

(vi) culturing the nuclear transfer embryo to a cell stage ranging from cleavage to blastocyst;

(vii) introducing one or more blastomeres into the perivitelline space of the cultured nuclear transfer embryo; and

(viii) culturing the resultant chimeric embryo to a sufficient stage of development such that it may be successfully implanted into a maternal recipient and result in a viable pregnancy.

33. The method of claim 32 wherein the chimeric non-human embryo is an ungulate.

34. The method of claim 33 wherein the ungulate is a cow.

35. A chimeric embryo produced according to the method of claim 32.

36. A chimeric embryo produced according to the method of claim 33.

37. A chimeric embryo produced according to the method of claim 34.

38. The method of claim 32 wherein the nuclear transfer embryo into which is introduced one or more blastomeres comprises from about 8 to 16 cells.

39. The method of claim 32 wherein the blastomeres are obtained from a similar staged fertilized embryo as the recipient nuclear transfer embryo.

40. The method of claim 32 wherein the blastomere are normal diploid blastomere or tetraploid blastomere.

41. The method of claim 32 wherein the blastomeres are transgenic.

42. The method of claim 32 wherein the culturing step (viii) is effected in vitro or in vivo.

43. The method of claim 1 wherein the embryonic stem cell which is introduced into the enucleated recipient oocyte is transgenic.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/13270

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12N 5/00, 15/00
US CL :424/7.1; 435/172.3, 240.2; 800/2
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/7.1; 435/172.3, 240.2; 800/2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	ANNUAL REVIEW OF NEUROLOGY, Volume 15, issued 1992, A. Zimmer, "Manipulating the genome by homologous recombination in embryonic stem cells", pages 115-137, see entire article.	1-43
Y	CHROMOSOMA, Volume 100, Number 5, issued 1991, M.S. Szollosi et al., "Chromatin behavior under influence of puromycin and 6-DMAP at different stages of mouse oocyte maturation", Abstract only, see entire Abstract.	1-43

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 23 MARCH 1995	Date of mailing of the international search report 06 APR 1995
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer BRIAN R. STANTON Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	DEVELOPMENTAL BIOLOGY, Volume 152, Number 1, issued 1992, D. Wiskramasinghe et al., "Centrosome phosphorylation and the developmental expression of meiotic competence in mouse oocytes", Abstract only, see entire Abstract.	1-43
Y	JOURNAL OF ASSISTED REPRODUCTION AND GENETICS, Volume 9, Number 4, issued 1992, P. DeSutter et al., "Parthenogenetic activation of human oocytes by puromycin", Abstract only, see entire Abstract.	1-43
Y	GAMETE RESEARCH, Volume 22, issued 1989, C.B. Ware et al., "Age dependence of bovine oocyte activation", pages 265-275, see entire article.	1-43
X --- Y	JOURNAL OF REPRODUCTION AND FERTILITY, Volume 98, Number 2, issued July 1993, Y. Tsunoda et al., "Nuclear transplantation of embryonic stem cells in mice", pages 537-540, see entire article.	1, 5-8, 23, 25-28 ---------- 2-4, 9-22, 24, and 29-43
Y	THERIOGENOLOGY, Volume 38, Number 2, issued August 1992, X. Yang et al., "Micromanipulation of mammalian embryos: Principles, progress and future possibilities", pages 315-335, see entire article.	1-43
X --- Y	ROUX'S ARCHIVE OF DEVELOPMENTAL BIOLOGY, Volume 201, issued 1992, S. Saito et al., "Bovine embryonic stem cell-like cell lines cultured over several passages", pages 134-141, see entire article.	1-43
Y	US, A, 5,096,822 (ROSENKRANS, JR. ET AL.) 17 MARCH 1992, see entire document.	1-43

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

Databases: APS, ANABSTR, AQUASCI, BIOBUSINESS, BIOSIS, BIOTECHABS, BIOTECHDS, CA, CABA, CAPREVIEWS, CEABA, CEN, CIN, CHACS, CJELSEVIER, CONFSCI, DISSABS, DRUGNL, EMBASE, FSTA, GENBANK, HEALSAFE, IFIPAT, JICST-E, JPNEWS, LIFESCI, MEDLINE, NTIS, OCEAN, PHIC, PHIN
Search terms: embryo?; stem; cell#; oocyte#; activat?; cold; cultur?; cation; calcium; magnesium; strontium; phosphor?; ionophor?; stauropurine; aminopurine; criaa; stice?/au; strelchenko?/au, scott?/au; jugella?/au; fus?; nuclear? transfer?; transplant?; dissaggre?; pig#; porcine; horse#; equin?; cow#; bovin?; antelop?; goat#; sheep; ovin?; ungulat?; electro?; sperm; oocyt?; calcium; ethanol; alcohol; kinase; inhibi?; serine; threonin?; phosphatase; pronas?; enucleat?; chimer?; transgen?; mouse; mice; murine

up four billion years ago, maybe another comet could create another LHB in the future.

However, ALH84001 has not yet told its full story. Measurements of carbon isotopes in several SNC meteorites were reported recently at a meeting in Houston. One of several possible interpretations of the unexpected data is that ALH84001 may not be from the same parent body as other SNCs — that is, not from Mars! Until samples are eventually returned by a mission to Mars, we will have to hope that Antarctic meteorite expeditions will find more SNC meteorites to further elucidate the red planet's early history. □

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EMBRYOLOGY

Lambing by nuclear transfer

Davor Solter

ALMOST all the exciting experiments that have increased our understanding of the genetic control of mammalian development have been done on the mouse. Attempts to extend this success to other mammalian species have generally foundered, so the cloning of identical sheep by nuclear transfer from cultured cells, described on page 64 of this issue by Campbell and co-workers[1], is cause for celebration. Aside from its intrinsic biological interest, this achievement opens up the possibility of manipulating the sheep's genes before cloning them.

The first description of a reliable nuclear transfer technique[2] was soon followed by reports of its limitations (reviewed in ref. 3). One reason why our understanding of the events inside the cell that follow nuclear transfer has been slow is that mice have proved surprisingly difficult to clone by this method.

In their experiment, Campbell *et al.* first derived a cell line from early sheep embryos. This cell line was totipotent, a condition in which cells retain their potential to differentiate into all the different cell types that make up the adult organism. Then, they carefully removed all chromosomal material from a recipient oocyte (see figure), fused this oocyte with a totipotent cell that had been through several passages in culture, and grew their manipulated embryos *in vivo*. The transplanted nuclei remained totipotent and embryos that developed to the morula/blastocyst stage were transferred to the uteri of ewes; several lambs were born and grew normally. It is quite fitting that this cloning experiment worked for sheep, because it was in sheep that nuclear transfer of cleavage-stage blastomere nuclei into enucleated eggs first resulted in the birth of normal lambs[4].

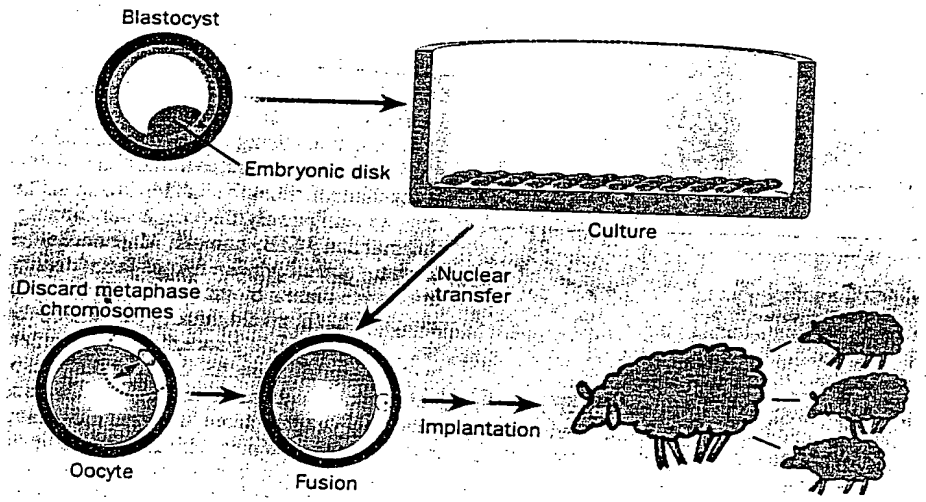
Once permanent cell lines with totipotent nuclei are established, there is in principle no limit to the genetic alterations that can be made: for example, transgenic strains of sheep and other agricultural animals could readily be established by deleting or modifying endogenous genes. But there are still several problems to be resolved before the technique can realize its full potential.

The overall success rate (live births compared to the number of manipulated embryos) is disappointingly low. The progress *in vivo* to the blastocyst stage and beyond needs to be improved. It is not clear whether the high failure rate is caused by the accumulation of assorted deleterious factors, perhaps caused by repeated manipulations, or whether it reflects heterogeneity within the cell line. The latter is unlikely as the success rate does not change with successive passages, but other, preferably cloned, totipotent cell lines need to be investigated to clarify this point. Knowledge of sheep (and cow and pig) genetics is essential for gene targeting, but at the moment this is rather limited.

Effective nuclear transfer, with development to term of the manipulated egg, must depend on adequate functional reprogramming of the donor nucleus. Macromolecules (messenger RNAs and proteins) stored in oocytes only support mammalian development for a relatively short time (as measured by the number of cell divisions), and the shorter this period, the less time there is for reprogramming. As the embryo undergoes more changes as it grows, the cells from older embryos will require more reprogramming time and will be less likely to reprogramme completely. Compatibility between the recipient cytoplasm and donor nucleus, still poorly understood, plays a part too.

The scant data available indicate that there are at least two factors that contribute to the success of nuclear transfer. First, ovulated oocytes are much better recipients than zygotes, either because there is more time for reprogramming or for some reason their cytoplasm is more suitable. For example, cytoplasmic factors necessary for chromosomal remodelling and genome activation may exist that are titrated out after fertilization, perhaps by binding to replicating DNA or by time-dependent degradation. Second, donor nuclei caught either in the G1 (refs 5, 6) or G0 (ref. 1) phase of the cell cycle result in much better development than those in late S or G2 phase (although there is at least one dissenting report[7]). This intuitively makes sense — it would seem to be easier to reprogramme a genome that is open for and undergoing replication.

Imprinting that results in functional differences in the male and female genome, and the need for both in successful development[8], could complicate the reprogramming of the genome after nuclear transfer. Setting aside the unlikely possibility of an absence of imprinting in sheep, the achievement of Campbell *et al.* indicates that the cells they used as nuclear donors



The process by which Campbell and colleagues[1] cloned lambs by nuclear transfer. Embryonic disks were cultured on a feeder layer and permanent cell lines established. The authors then enucleated recipient oocytes by removing a small amount of cytoplasm containing the metaphase plate, and fused them with a single cell from the totipotent cell line. Reconstructed embryos were transferred to temporary recipient ewes and their development was assessed after seven days. Finally, morulae and blastocysts were implanted in other ewes and allowed to develop to term.

retained enough of the functional imprint to support development. This explanation is borne out by the paternal X chromosome of mouse embryonic stem cells, which keeps the imprinting necessary to ensure its preferential inactivation in extraembryonic membranes[9]. On the other hand, a partial loss of imprinting in the cells used by Campbell *et al.* might have contributed to the low rate of development of manipulated embryos.

The benefits of being able to clone valuable farm animals are obvious, although so far the technical and biological problems have limited the potential impact. The results of Campbell and colleagues, the production of calves as a result of nuclear transfer from cultured inner-cell mass cells[10], and the isolation of embryonic stem cell lines from primates[11], are all indicators that the technical know-how is to hand to produce clones of different mammals. Cloning mammals from adult cells will be considerably harder, but can no longer be considered impossible: it might be a good idea to start thinking how we are going to make use of such an option. □

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DEVELOPMENTAL BIOLOGY

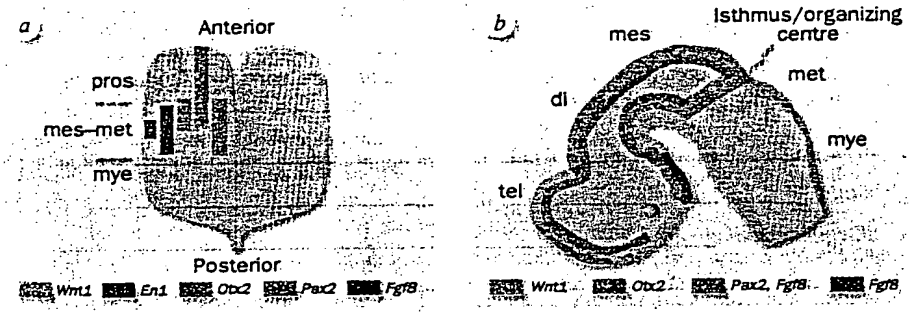
The brain organization

Siew-Lan Ang

THE paper by Crossley *et al.* on page 66 of this issue[1] takes us one step forward in answering a central question in developmental biology — how the regional identity of the vertebrate brain is specified along the anterior/posterior axis. This elegant study shows that *Fgf8*, a member of the fibroblast growth factor family, has a key part in midbrain development.

The first phase in the induction and early regionalization of the neural tube, the structure that develops into the brain and spinal cord, has been extensively studied in the frog, chick and more recently the mouse[2–4]. It involves inductive interactions during gastrulation between two germ layers, mesoderm and ectoderm. These early interactions subdivide the front part of the neural tube into broad regions, consisting of the forebrain (prosencephalon), midbrain and anterior hindbrain (mesencephalon–metencephalon; mes–met) and posterior hindbrain (myelencephalon), that are distinguishable by the expression of region-specific genes. In particular, the mes–met region is characterized by the overlapping expression of the homeobox-containing genes *En1* and *Otx2*, the paired-box-containing gene *Pax2*, and the signalling molecules *Wnt1* and *Fgf8* (refs 5–7; *a* in the figure).

During a second phase of patterning of the mes–met region, the mesencephalon and metencephalon acquire distinct fates, marked by progressive restriction of *Otx2* and *Wnt1* to the mesencephalic side and of *Pax2* and *Fgf8* to the metencephalic side of a constriction between these two territories called isthmus[5–7] (*b* in the figure). Mesencephalon and metencephalon will subsequently differentiate into midbrain and cerebellum respectively. Experiments involving the transplantation of embryonic brain tissue in chicks have shown that this second phase of patterning could depend on signals working in the plane of the neural tube[8,9]. In particular, these studies have revealed a central role for cells of the isthmus. When isthmic tissue is transplanted in the caudal forebrain, the host tissue is transformed into an ectopic midbrain that is the mirror image of the normal midbrain[10]. The isthmic tissue thus appears to serve as an organizing centre, producing a signal that can induce and pattern midbrain tissue.



Expression of the *Wnt1*, *En1*, *Otx2*, *Pax2* and *Fgf8* genes at an early stage (embryo day 8, E8.0) and a late stage (E9.5) of mouse brain patterning. *a*, Dorsal neural plate at E8.0. Overlapping areas of expression of *Wnt1*, *En1*, *Pax2*, *Otx2* and *Fgf8* in the mes–met region are indicated. The domain of *Fgf8* expression shown corresponds to its expression at E8.5 according to Crossley and Martin[7], as earlier expression of *Fgf8* in the neural plate has not been described. The posterior limit of *Otx2* is fuzzy at this stage, as depicted by fading colour at its caudal end. *b*, Mouse brain at E9.5 in lateral view. *Wnt1* and *Otx2* are expressed in the mesencephalic side, whereas *Fgf8* and *Pax2* are expressed in the metencephalic side of the isthmus. *Pax2* expression in the optic vesicle is not shown. pros, prosencephalon; mes, mesencephalon; met, metencephalon; mye, myelencephalon; tel, telencephalon; di, diencephalon. (*a* and *b* adapted from refs 5 and 7 respectively.)

The demonstration of an organizing centre at the isthmus of the met–mes region, and of adjacent expression of *Wnt1* and *Fgf8* within this region (*b* in the figure), aroused great interest. Crossley *et al.*[1] have now investigated the role of *Fgf8* in development of the mes–met region using grafting experiments in chicks. First, they showed that *Fgf8* is expressed in the isthmic region of chick embryos at the right time to be involved in the development of the organizing centre. They then implanted beads soaked in recombinant FGF8 protein into the caudal forebrain of chick embryos and made the striking observation that FGF8 produced the same effect as transplantation of isthmic tissue — in 17 out of 18 cases, treatment with the beads resulted in the induction of an ectopic midbrain with a mirror-image polarity compared to the normal midbrain. FGF8 also induced ectopic expression of *Wnt1*, *En2* and *Fgf8* itself, three genes that are normally expressed in the mes–met region. Together, these results demonstrate that FGF8 can induce a complete midbrain by establishing an ectopic isthmus-like organizing centre in the forebrain.

From analysis of mouse mutants, it is clear that WNT1 also itself functions in the successive phases of patterning of the mes–met region. For instance, embryos

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Sheep cloned by nuclear transfer from a cultured cell line

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Nuclear transfer has been used in mammals as both a valuable tool in embryological studies[1] and as a method for the multiplication of 'elite' embryos[2–4]. Offspring have only been reported when early embryos, or embryo-derived cells during primary culture, were used as nuclear donors[5,6]. Here we provide the first report, to our knowledge, of live mammalian offspring following nuclear transfer from an established cell line. Lambs were born after cells derived from sheep embryos, which had been cultured for 6 to 13 passages, were induced to quiesce by serum starvation before transfer of their nuclei into enucleated oocytes. Induction of quiescence in the donor cells may modify the donor chromatin structure to help nuclear reprogramming and allow development. This approach will provide the same powerful opportunities for analysis and modification of gene function in livestock species that are available in the mouse through the use of embryonic stem cells[7].

The cells used in these experiments were isolated by microdissection and explantation of the embryonic disc (ED) of day 9 *in vivo* produced 'Welsh mountain' sheep embryos. The line was established from early passage colonies with a morphology like that of embryonic stem (ES) cells. By the second and third passages, the cells had assumed a more epithelial, flattened morphology (Fig. 1*a*) which was maintained on further culture (to at least passage 25). At passage 6, unlike murine ES cells they expressed cytokeratin, and nuclear lamin A/C which are markers associated with differentiation[8]. This embryo-derived epithelial cell line has been designated TNT4 (for totipotent for nuclear transfer).

The development of embryos reconstructed by nuclear transfer is dependent upon interactions between the donor nucleus and the recipient cytoplasm. We have previously reported the effects of the cytoplasmic kinase activity, maturation/mitosis/meiosis promoting factor (MPF), on the incidence of chromosomal damage and aneuploidy in reconstructed embryos and established two means of preventing such damage[9]. First, the effects of the donor cell-cycle stage can be overcome by transferring nuclei after the disappearance of MPF activity by prior activation of the recipient enucleated MII oocyte[9,10]. Using this approach we obtained the birth of lambs by nuclear transfer during establishment of the cell line (up to and including passage 3). On subsequent culture (passages 6 and 11) no development to term was obtained (see Table 1). From these numbers we cannot conclude that development to term will not be obtained using this method. The lack of development of some control embryos is thought to relate to an infection in the oviduct of the temporary recipient ewe from which 6 were recovered.

An alternative means of avoiding damage due to the activity of MPF is to transfer diploid nuclei into metaphase II oocytes that have a high level of MPF activity[9]. The availability of TNT4 cells allows this approach to be used. In this study a synchronous population of diploid donor nuclei was produced by inducing the cells to exit the growth cycle and arrest in G0 in a state of quiescence. In the presence of a high level of MPF activity the transferred nucleus undergoes nuclear membrane breakdown and chromosome condensation. It has been argued[11] that the developmental potential of reconstructed embryos depends upon the "reprogramming of gene expression" by the action of cytoplasmic factors and that this might be enhanced by the prolongation of this period of exposure. To assess these effects donor cells were fused to oocytes either (1) 4–8 h before activation 'post-activated' or (2) at the time of activation 'fusion and activation' or (3) to pre-activated oocytes 'preactivated'.

During these studies *in vivo* ovulated metaphase arrested (MII) oocytes were flushed from the oviduct of 'Scottish blackface' ewes. The methodology used was as previously described[10] with the following exceptions; oocytes were recovered 28–33 h after injection of gonadotropin-releasing hormone (GnRH), calcium/magnesium-free PBS containing 1.0% FCS was used for all flushing, and recovered oocytes were transferred to calcium-free M2 medium[12] containing 10% FCS and were maintained at 37 °C in 5% CO_2 in air until use. As soon as possible after recovery oocytes were enucleated and embryos reconstructed. At 50–54 h

TABLE 1 Development using unsynchronized TNT4 cells

Donor cell type	Number of morula and blastocysts/total embryos (%)	Number of lambs/ embryos transferred
	October 1993–February 1994	
16 cell	6/11 (27.3)	2/6
ED cell	1/15 (6.7)	0/1
ED P1	4/19 (21.0)	1/4
ED P2	1/11 (9.1)	1/1
ED P3	2/36 (5.5)	2/2
	October–December 1994	
16 cell	14/28 (50.0)	0/14
TNT4 P6	9/98 (9.2)	0*/9
TNT4 P11	10/92 (10.9)	0/10

Development of ovine embryos reconstructed by nuclear transfer of unsynchronized cells during isolation and after establishment of the TNT4 line to enucleated preactivated ovine oocytes. P, Passage number; ED, embryonic disc. For embryo reconstruction, donor oocytes were placed into calcium-free M2 containing 10% FCS, 7.5 μg ml^{-1} Cytochalasin B (Sigma) and 5.0 μg ml^{-1} Hoechst 33342 (Sigma) at 37 °C for 20 min to aspirate. A small amount of cytoplasm enclosed in plasma membrane was removed from directly beneath the 1st polar body using a glass pipette (~20 μm tip external diameter). Enucleation was confirmed by exposing this karyoplast to ultraviolet light and checking for the presence of a metaphase plate. At 34–36 h after GnRH injection enucleated oocytes were activated. Following further culture for 4–6 h in TC199, 10% FCS a single cell was fused. All activations and fusions were accomplished as previously described[10,17] in 0.3 M mannitol, 0.1 mM $MgSO_4$, 0.0005 mM $CaCl_2$[17]. For activation a single DC pulse of 1.25 kV cm^{-1} for 80 μs and for fusion an AC pulse of 3 V for 5 s followed by 3 d.c. pulses of 1.25 kV cm^{-1} for 80 μs were applied. All oocyte/cell couplets were cultured in TC199, 10% FCS 7.5 μg ml^{-1} Cytochalasin B (SIGMA) for 1 h following application of the fusion pulse and then in the same medium without Cytochalasin until transferred to temporary recipient ewes. Reconstructed embryos were cultured in the ligated oviduct of a recipient 'blackface' ewe until day 7 after reconstruction. All morula and blastocyst stage embryos were transferred to synchronized recipient blackface ewes for development to term.

* A single pregnancy was established but subsequently lost at about 70–80 days.

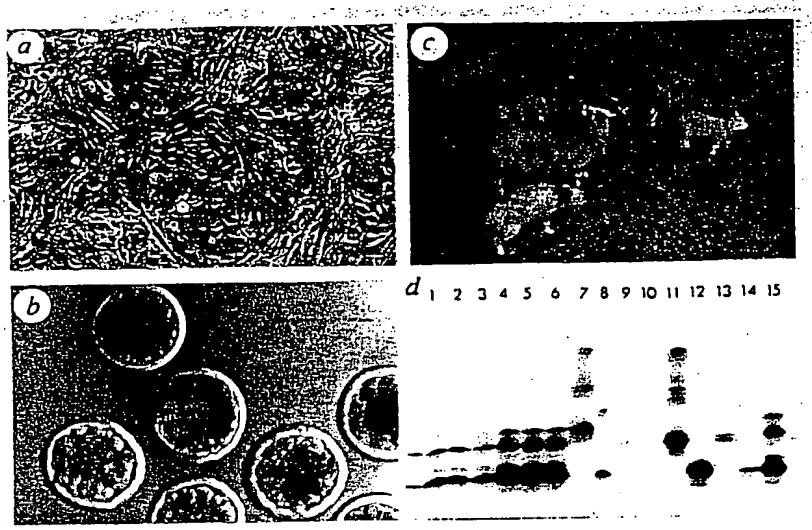

FIG. 1 Production and characterization of the TNT4 cell line and the offspring produced by nuclear transfer from TNT4 cells. *a*, Morphology of the TNT4 cell line at passage 6. *b*, Group of embryos including a single blastocyst on day 7 after reconstruction. *c*, Group of three Welsh mountain lambs produced by nuclear transfer with surrogate Scottish blackface ewes. *d*, Autoradiogram showing the alleles generated following amplification of the microsatellite FCB266 (ref. 18). Lanes 1–6 are from, respectively, TNT4 cells and the five lambs generated by nuclear transfer. Both lambs and cells display an identical pattern, revealing 2 alleles (arrowed) at 114 and 125 bp. Lanes 7–15, nine randomly chosen Welsh mountain sheep, none of whom show an identical pattern to the nuclear transfer group. Lambs and TNT4 cells were also identical at six further microsatellite loci: MAF33, MAF48, MAF65, MAF209, OarFCB11, OarFCB128, OarRCB304 (data not shown). The nine unrelated random control animals showed extensive variation at all of these loci.

METHODS. Groups of 4–6 microdissected embryonic discs were cultured on feeder layers of mitotically inactivated primary murine fibroblasts in Dulbeccos Modified Eagles medium (GIBCO) containing 10% fetal calf serum, 10% newborn serum and supplemented with recombinant human leukaemia inhibition factor (LIF). After 5–7 days of culture, expanding discs were treated with trypsin and passaged onto fresh feeders yielding 4 similar lines. At passage 12 of the 2*n* chromosome complement of 54 was observed in 31 of 50 spreads, the remaining aneuploid spreads are thought to be artefacts of preparation. For microsatellite analysis genomic DNA was extracted from whole blood, tissue culture cells or fetal tissues using a puregene DNA isolation kit (Gentra Systems Inc., Minneapolis, USA). The PCR analysis of microsatellites was carried out using an end-labelled primer ([γ-^{32}P]ATP). All other aspects of labelling and thermal cycling conditions were as described elsewhere[17].

after GnRH injection, reconstructed embryos were embedded in agar and transferred to the ligated oviduct of dioestrus ewes. After 6 days the embryos were retrieved and development assessed microscopically (see Fig. 1*b*).

The development of embryos reconstructed using quiescent TNT4 cells and 3 different cytoplast recipients is summarized in Table 2. No significant difference was observed in the frequency of development with high and low passage number donor cells or with cytoplast recipient type used (results were analysed by the marginal model in ref. 13). All embryos that had developed to the morula/blastocyst stage were transferred as soon as possible to the uterine horn of synchronized final recipient ewes for development to term. Recipient ewes were monitored for pregnancy by ultrasonography. Ewes that were positive at day 35 were classified as pregnant (Table 3). A total of eight fetuses were detected in seven recipient ewes including a single twin pregnancy. A total of five phenotypically female Welsh mountain lambs were born from the Scottish blackface recipient ewes (Fig. 1*c*). Two of these lambs died within minutes of birth and a third at 10 days; the remaining two lambs are apparently normal and healthy (8–9 months old). Of the remaining 3 fetuses, one was lost at about 80 days of gestation, and a second was lost at 144 days of gestation. The third fetus was thought to be a twin pregnancy and was either misdiagnosed or lost at an unknown time. Microsatellite analysis of the cell line, fetuses and lambs showed that all of the female lambs were derived from a single cell population (Fig. 1*d*).

TABLE 2 Development to morula and blastocyst stage of ovine embryos reconstructed using quiescent TNT4 cells and 3 different cytoplast recipients (January–March 1995)

Experiment number	Cytoplast type: TNT passage number	Number of morulae and blastocysts/total number of embryos recovered (%): Post-activated	Activation and fusion	Preactivated
1	6	4/28	6/32*	–
2	7	1/10	1/26*	–
3	13	0/2	–	2/14
4	13	0/14	0/11	–
5	11	1/9	–	0/9
6	11	1/2	9/29***	–
7	12	–		6/45*
8	13	3/13*		–
Total		10/78 (12.8%)	16/98 (16.3%)	8/68 (11.7%)

Development to the morula and blastocyst stage of ovine embryos recovered on day 7 after reconstruction by nuclear transfer of quiescent TNT4 cells at different passages into 3 cytoplast recipients. To induce quiescence, TNT4 cells were plated into feeder layers in 29-cm^2 flasks (GIBCO) and cultured for 2 days, the semiconfluent exponentially growing cultures were then washed three times in medium containing 0.5% FCS and cultured in this low-serum medium for 5 days. Embryos were reconstructed using preactivated cytoplasts as previously described (Table 1) and by two other protocols. (1) post-activation, as soon as possible after enucleation a single cell was fused to the cytoplast in 0.3 M mannitol without calcium and magnesium, to prevent activation. Couplets were washed and cultured in calcium-free M2, 10% FCS at 37 °C, 5% CO_2 for 4–8 h. Thirty minutes before activation the couplets were transferred to M2 medium, 10% FCS containing 5 μM Nocodazole (SIGMA). Following activation the reconstructed zygotes were incubated in medium TC199, 10% FCS, 5.0 μM Nocodazole for a further 3 h. (2) Preactivation, at 34–36 h after GnRH injection a single cell was fused to an enucleated oocyte. The same pulse also induced activation of the recipient cytoplast. All activations and fusions were accomplished as described in Table 1 unless otherwise stated.

* Denotes number of pregnancies following transfer of morula and blastocyst stage embryos to synchronized final recipient ewes.

TABLE 3 Induction of pregnancy and further development following transfer of morula and blastocyst stage embryos reconstructed from quiescent TNT4 cells

Cytoplast type	Post-activated	Activation and fusion	Preactivated
Total number of morula and blastocyst stage embryos transferred	10	16	8
Total number of ewes	6	9	4
Number of pregnant ewes (%)	1 (16.7)	5 (55.5)	1 (25.0)
Number of fetuses/total embryos transferred (%)	2/10 (20.0)	5/16 (31.25)	1/8 (12.5)
Number of live births	1	3	1
Passage number of cells resulting in offspring	1 × P11	1 × P6, 2 × P11	1 × P13

Induction of pregnancy following transfer of all morula/blastocyst stage reconstructed embryos to the uterine horn of synchronized final recipient blackface ewes. The table shows the total number of embryos from each group transferred, the frequency of pregnancy in terms of ewes and embryos (in the majority of cases 2 embryos were transferred to each ewe and a single twin pregnancy was established (using the 'post-activated' cytoplast)) and the number of live lambs obtained.

Because of the seasonality of sheep a direct comparison of all of these methods of embryo reconstruction has not yet been made. The success of the later studies may be due to a number of factors. First, quiescent nuclei are diploid and therefore the cell-cycle stages of the karyoplast and cytoplast in both the 'post-activation' and 'fusion and activation' methods of reconstruction are coordinated. The preactivated cytoplast will accept donor nuclei from G0, G1, S and G2 cell-cycle phases. Second, the G0 phase of the cell cycle has been implicated in the differentiation process and the chromatin of quiescent nuclei has been reported to undergo modification[14]. As a result the chromatin of quiescent donor nuclei may be more readily modified by oocyte cytoplasm. The TNT4 cells resemble several cell lines derived previously in sheep[15] and also pigs[16]. It remains to be determined whether comparable development is obtained with other such lines or other cell types. At the present time we are unable to differentiate the mechanisms involved and report that the combination of nuclear transfer and cell type described here support development to term of cloned ovine embryos from cells that had been in culture through up to 13 passages. As cell-cycle duration was about 24 h, this period of culture before nuclear transfer would be sufficient to allow genetic modification and selection if procedures comparable to those used in murine ES cells can be established.

The production of cloned offspring in farm animal species could provide enormous benefits in research, agriculture and biotechnology. The modification by gene targeting and selection of cell populations before embryo reconstruction coupled to the clonal origin of the whole animal provides a method for the dissemination of rapid genetic improvement and/or modification into the population □

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Midbrain development induced by FGF8 in the chick embryo

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VERTEBRATE midbrain development depends on an organizing centre located at the isthmus, a constriction in the embryonic mid/hindbrain region[1–3,28]. Isthmic tissue grafts transform chick caudal forebrain into an ectopic midbrain that is the mirror image of the normal midbrain[4]. Here we report that FGF8 protein has the same midbrain-inducing and polarizing effect as isthmic tissue. Moreover, FGF8 induces ectopic expression in the forebrain of genes normally expressed in the isthmus, suggesting that the ectopic midbrain forms under the influence of signals from a new 'isthmus-like' organizing centre induced in the forebrain. Because *Fgf8* itself is expressed in the isthmus, our results identify FGF8 as an important signalling molecule in normal midbrain development.

Fgf8 is expressed in the isthmus of the developing mouse brain[5–8]. Because FGF8 has inducing activity in another developmental system (the limb[9]), we sought to determine whether FGF8 provides the midbrain-inducing activity of an isthmus graft in the chick. We first confirmed that *Fgf8* is expressed in the chick isthmus (Fig. 1*a*). Next, we determined the effects of implanting a bead soaked in recombinant FGF8 (FGF8-bead) into the caudal diencephalon (prosomere 2, p2, as defined in ref. 10; Fig. 1*b*) of chick embryos at stages 9–12 (ref. 11). An early effect of isthmus grafts is induction in the host neuroepithelium of *Engrailed*-2 (*En2*) expression[1,4], an early marker of mes/rhombencephalic development[12–14]. When an FGF8-bead was implanted, ectopic *En2* RNA was detected caudal to the zona limitans intrathalamica (ZL), a transverse boundary separating dorsal and ventral thalamus anlagen (p2/p3 boundary[10]), in all embryos assayed 22–26 h later ($n = 10$; Fig. 1*c*). Control beads soaked in phosphate-buffered saline (PBS-beads) did not induce *En2* expression ($n = 11$; not shown).

In experimental embryos surviving to E5–E16 (stages 25–42) the diencephalon caudal to the ZL (p1 and p2; ref. 10) was transformed from its normal fate of rostral pretectum and dorsal thalamus to ectopic midbrain ($n = 17/18$; Fig. 2). Control embryos implanted with PBS-beads that survived to E4–E10

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Production of calves by transfer of nuclei from cultured inner cell mass cells

(bovine embryonic stem cells/nuclear transfer/totipotency)

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ABSTRACT **We report here the isolation and *in vitro* culture of bovine inner cell mass (ICM) cells and the use of ICM cells in nuclear transfer to produce totipotent blastocysts that resulted in calves born. Of 15 cell lines represented in this study, 13 were derived from immunosurgically isolated ICM of 3 *in vitro* produced day 9–10 bovine blastocysts, while 2 lines were derived from single blastocysts. Approximately 70% of attempted cell lines became established cell lines when started from 3 ICMs. The ability to establish cell lines was dependent on the number of ICMs starting the line. Sire differences were noted in the ability of ICMs to establish cell lines and to form blastocysts. The cell lines were cultured as a low cell density suspension in the medium CR1aa plus selenium, insulin, and transferrin (SIT) and 5% fetal calf serum (FCS) for 6–101 days before use in nuclear transfer, at which time some had multiplied to more than 2000 cells. If allowed to aggregate, cells of established cell lines formed embryoid bodies. A total of 659 nuclear transfer clones were made by fusing the ES cells into enucleated oocytes with polyethylene glycol; 460 of these fused, based on cleavage (70%). After culture of the clones for 7 days *in vitro* in CR1aa/SIT/5% FCS, 109 (24%) of those fused became blastocysts. Thirty-four blastocysts were transferred into uteri of 27 cows, and 13 cows (49%) became pregnant. Four of the 13 cows gave birth to 4 normal calves. DNA typing showed the calves to be derived from the respective sires of the cell lines. The calves were derived from cultures of less than 28 days.**

The isolation and multiplication in culture of totipotent embryonic stem (ES) cells have value in providing a large population of identical cells for use by nuclear transfer to produce clonal offspring (1). ES cells also provide a mechanism for gene transfer by transfection, infection, or injection of genes into the cells (2–6). After insertion of a selectable marker, the transgenic cells can be separated and used either by chimerization into a blastocyst or through use as donor cells in nuclear transfer to produce transgenic offspring (5–7). In addition, homologous recombination techniques can be used with cultured ES cells to add or delete genes at specific sites in the genome (8–11).

All of the above have been accomplished only with ES cells of mice (6, 12). In mice, no offspring from presumed totipotent ES cells have been produced by conventional nuclear transfer (12, 13), although offspring were produced when mouse ES cells were chimerized with tetraploid mouse embryos (14).

For domestic animals, morphological identification of putative ES cells has been published (15–18). Pluripotency has been demonstrated for ES cells of swine (17–19), cattle (17, 20, 21), and sheep (19). Injection of newly isolated blastocyst inner cell mass (ICM) cells into other blastocysts has produced chimeric offspring in sheep (22) and cattle (23). Noncultured ICM cells appear to be totipotent as evidenced by blastocyst formation, pregnancies, and offspring after transfer into enucleated oocytes in rabbits (24), sheep (25), and cattle (26).

Cultured cells with ES cell characteristics have been transferred into bovine oocytes initially with the resulting 5-day cultured embryos surviving only to the 8-cell stage (27). More recently, bovine cell lines derived from ICM (20) or morulae (21) have produced pregnancies by nuclear transfer, which fail in the first trimester. Calves have been born from chimeric embryos but the ES cell contribution is as yet unknown. One chimeric fetus was ES cell positive (20). There are no published reports in domestic species that cultured ICM or putative ES cells are totipotent, as evidenced by offspring derived totally from these cells (6, 12, 17, 19–21, 27).

Most attempts to isolate and culture ICM cells have been based on or adapted from the original methods of Evans and coworkers for mice (2, 17). In general, these methods involve separation of blastocyst ICM from trophoblast trophectoderm cells by immunosurgery followed by isolation of cells with stem cell morphological characteristics from ICM cells as they plate down on a fibroblast feeder layer. The putative stem cells are then maintained as a colony on a monolayer of fibroblast cells with differentiation-inhibiting activity, leukemia inhibitory factor, buffalo rat liver (BRL) cells, or BRL conditioned medium added to inhibit differentiation. This system has allowed culture of pluripotent cells that can become embryoid bodies. Aggregated sheets of cells develop cellular beating heart activity. However, only in mice has it allowed demonstration of or maintenance of totipotency of the cultured cells [reviewed by Stewart (6) and Anderson (12)]. It has been suggested that these mouse-derived differentiation-inhibiting agents do not adequately prevent differentiation of stem cells in species other than rodents (12).

We report here the isolation and short-term *in vitro* culture of bovine ICM cells by using a different approach to prevent differentiation. These cells were used in nuclear transfer to produce blastocysts that resulted in the birth of normal calves. This result provides evidence of totipotency of cultured ICM cells in mammalian species other than mouse.

MATERIALS AND METHODS

All embryos used in this experiment were *in vitro* derived from slaughterhouse ovaries and frozen semen by the methods described by Sirard *et al.* (28), Parrish *et al.* (29), and Rosenkrans and First (30). Oocyte maturation was in TC199 containing 10% fetal calf serum (FCS) and 0.5 μg of NIH ovine luteinizing hormone (NIADDK-OLH-25) per ml. Oocytes were fertilized with sperm from any one of five different bulls

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Abbreviations: ES cells, embryonic stem cells; SIT, selenium, insulin, and transferrin; FCS, fetal calf serum; ICM, inner cell mass.

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with sperm concentration and heparin dose adjusted for each bull. At 40–48 hr postfertilization, embryos were manually stripped of all cumulus cells and extraneous sperm by repeated pipetting through a 190-μm fire-polished pipette. Subsequent embryo culture was carried out in a defined medium called CR1aa (30) for 7–8 days at 39°C in 5% CO_2/95% air with high humidity until the embryos had hatched or were fully expanded, after which they were subjected to immunosurgery. Embryos were first washed in 3 ml of TL Hepes with polyvinylpyrrolidone (Sigma; PVP-40) (1 mg/ml) and polyvinyl alcohol (Sigma; p8136) (1 mg/ml) and then washed through four or five CO_2 equilibrated microdrops (50 μl) of CR1aa, polyvinylpyrrolidone, and polyvinyl alcohol under paraffin oil.

Rabbit anti-bovine antibody (1:10 dilution; Sigma; B8270) was added at a 1:10 dilution for a final concentration of 1:100. Embryos were returned to the 39°C incubator for 30 min. The embryos were removed from the incubator and again washed through four or five fresh microdrops of medium. Then guinea pig complement (Sigma; S-1639) was added to the embryos at a 1:10 dilution from a 1:500 diluted stock for a final dilution of 1:5000. While in the presence of complement, the zonae pellucidae were removed by manual pipetting through a non-fire-polished 150-μm pipette tip. The remaining ICMs were washed and then one to three ICMs per 10-μl drop of the medium CR1aa plus SIT (sodium selenite, insulin, and transferrin; Sigma; I 1884) were placed under paraffin oil. Within 5 days, the ICMs started disassociating from a ball of cells into individual free-floating cells. At this time, the ball of cells was mechanically disaggregated by a micromanipulation needle. The medium was changed every 2–3 days by aspiration and replaced with fresh CO_2-equilibrated CR1aa with SIT. The addition of 5% FCS to the medium was beneficial in reducing the "stickiness" of these cells, allowing easier handling during micromanipulation. The ICM cells were maintained as disassociated cells in suspension culture for periods ranging from 1 week to 2 months, depending on the experimental protocol. The culture conditions were derived through a series of experiments comparing the effects of various growth factors, media, and medium supplements on cell maintenance and growth rates. Cell viability was determined by staining with propidium iodide. All embryos used to make cell lines were derived from embryos cultured in the CR1aa/SIT/5% FCS medium.

The cultured ICM cells were used as nuclear donor cells in nuclear transfer. Recipient oocytes were matured *in vitro* (29) and stripped of cumulus 16–18 hr after initiation of oocyte maturation, using hyaluronidase at a concentration of 2 mg/ml and a fire-polished pipette. Oocytes were selected for the presence of polar bodies and returned to maturation medium for another 2–4 hr. Nuclear transfer was begun ≈20 hr after these metaphase II oocytes were placed into culture. Manipulation was performed with a Nikon Diaphot microscope equipped with Hoffman optics and Narishige micromanipulators. Manipulation was done in culture dishes in which microdrops of medium were arranged with each dish containing both 100-μl drops (TL Hepes with Ca^{2+} and Mg^{2+}) in which the oocytes were placed and 20-μl drops (TL Hepes with Ca^{2+} and Mg^{2+} and 20–50% FCS) to one side containing the cultured ICM cells. This was done to prevent the cells from sticking to the oocytes and to prevent mistaking ICM cells with any remaining cumulus cells. Approximately 10 ICM cells were aspirated into the transfer pipette, and then the tips were moved to the drop containing the oocytes. The cells were drawn higher into the pipette to allow space for enucleation of the oocyte. The oocyte was positioned on a holding pipette so that the polar body was toward the transfer tip. A small amount of cytoplasm from the region directly beneath the polar body and the polar body were removed. The transfer tip was retracted from the zona and the cytoplasm was ejected. The tip was reinserted through the same hole and an ICM cell was deposited beneath the zona. The cell was pressed against the plasma membrane, where it stuck firmly between the zona and plasma membrane. Due to the extreme stickiness of the cells, transfer pipettes were changed frequently. Nuclear (ICM cell) transfer was completed by 24 hr postmaturation, and the unfused units were placed in CR1aa medium overnight. All fusions were done with oocytes 42 hr postfollicular removal.

Fusion proved to be a difficult problem because of the disparate sizes of the cells to be fused. The ICM cells ranged in size from 15 to 25 μm, and the enucleated oocytes were ≈140 μm. Except for Table 4, in which recent experiments (bulls F–I) used electrofusion, fusions were with polyethylene glycol (PEG). The fusion protocol used PEG (M_r 1300–1600; Sigma) 1:0.25 g/ml in Ca^{2+}- and Mg^{2+}-free TL Hepes with polyvinyl alcohol (1 mg/ml) for 45 sec followed by a 1:1 dilution in the same medium for 1 min, another 1:1 dilution for 2 min, and then a final 1:1 dilution for 2–3 min. The most reliable PEG was from Boehringer Mannheim (PEG 1500). A 15-min culture in TL Hepes containing 20% FCS allowed membranes to return to their normal appearance. To activate the ooplasm, the embryos were washed through Ca^{2+}- and Mg^{2+}-free TL Hepes and then exposed to 5 mM ionomycin (Calbiochem) in 1 ml of medium for 45 sec. This was followed by another 15-min culture in TL Hepes containing 20% FCS, after which embryos were returned to CR1aa medium for further maturation.

RESULTS

To prevent differentiation, ICM cells were cultured in suspension at a concentration sufficiently low (1000–1500 cells per 10-μl drop) so that cell aggregation and differentiation did not occur. Several differentiation-inhibiting and mitotic factors were tested in various media combinations for their ability to promote prolonged mitotic activity of ICM cells cultured in loose suspension. Only media consisting of CR1aa plus SIT and either glucose, rifampicin, laminin, or 5% FCS supported mitosis through 2 weeks of culture. Of these, only CR1aa plus SIT plus 5% FCS allowed mitosis and continued proliferation of ICM cells for 4 weeks. ICM cells from day 9 and 10 bovine blastocysts multiplied in culture when cultured in CR1aa plus SIT and 5% FCS with some lines reaching 2000 cells after 2 weeks of culture. These cells have the appearance of mouse ES cells, being small cells with large nuclei, little cytoplasm, and prominent nucleoli (Fig. 1). When

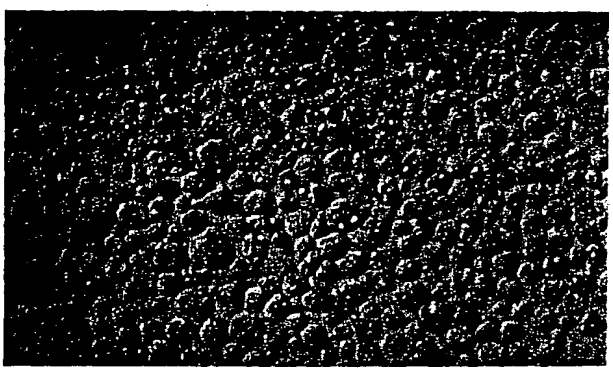

FIG. 1. Bovine ES cells. Cell population in 10-μl microdrops varied from 200 to 2000 cells within 2 weeks of culture. Individual lines were subcultured at 1000–1500 cells per microdrop because embryoid bodies formed when cell population densities exceeded 1000 cells. Note that the nucleus constitutes most of the volume of each cell, the presence of two or three nucleoli per cell, and the large round cells that will soon divide.

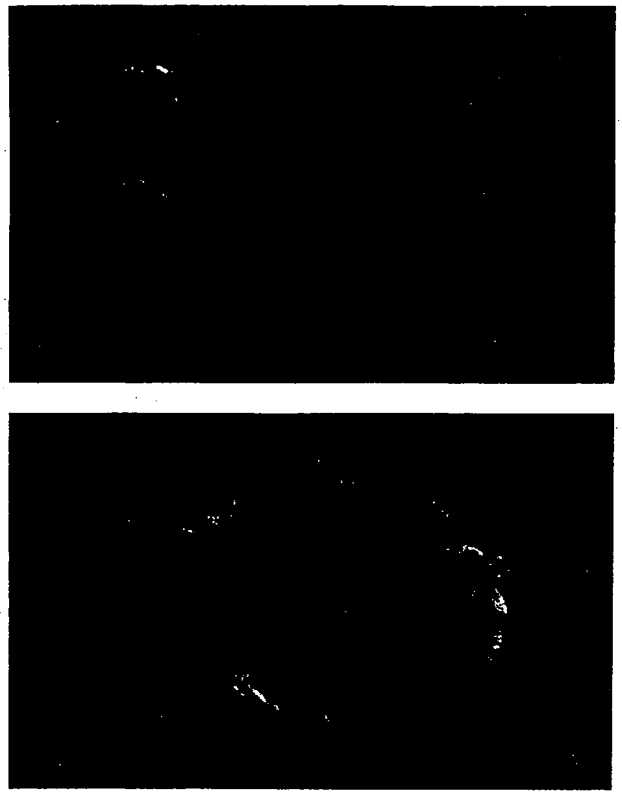

FIG. 2. Embryoid bodies resulting from high cell population density. (*Upper*) Bovine embryoid bodies, two simple and one complex. (*Lower*) Bovine complex embryoid body.

removed from nondifferentiating conditions and allowed to aggregate, the cultured cells formed embryoid bodies (Fig. 2).

Table 1. Effect of number of ICM cells starting a culture on ICM cell survival

No. of ICMs starting cell line	No. of cell lines	
	Started	Surviving at 1 month
1	159	0
3	241	170 (70.54%)

These embryoid bodies do not appear to differ morphologically from mouse embryoid bodies.

The ability of ICM cells cultured in loose suspension microdrops to establish and maintain a proliferating population of ICM cells appears to be dependent on the number of ICMs used to establish the culture (Table 1). ICM cultures derived from three ICMs from two sires established cultures that proliferated, whereas none of the cultures started from one ICM survived to 10 days and even the surviving cultures had a low proliferation rate, with most dying by 1 month. Occasionally, a single ICM initiated a cell culture as the two shown in Table 2. While one cell line, line six, was maintained for 101 days, most ICM lines derived from pooled embryos lost life and nuclear staining after 3 weeks of culture. Live/dead staining at 3 weeks with calcein AM (live) or ethidium homodimer (dead) showed ≈80% live cells (green) and 20% dead (red) and dying (orange-yellow) cells, whereas, at 5 weeks, nearly 80% of the cells were dead or dying.

One way of accurately determining totipotency of embryonic cells is to fuse the cell in question into an enucleated metaphase II oocyte. We report in Table 2 results from derivation and use of cells from 15 bovine ICM cell lines in nuclear transfer. The cell lines ranged from 6 to 101 days of culture at the time of nuclear transfer. A total of 659 embryos (clones) were made by nuclear transfer. After culture for 7 days *in vitro* in CR1aa and SIT plus 5% FCS, 109 became blastocysts (16.6%); of those cleaving, 25% became blastocysts. Each cell line was derived from the ICM of 3 blastocysts except cell lines 14 and 15, which were each derived from the ICM of a single blastocyst. The efficiency of establishing stem cell cultures from ICM cells and the ability of the oocyte ICM cell fusion product to become a blastocyst

Table 2. Use of loose suspension cultured ICM cells as donors of nuclei in nuclear transfer to produce blastocyts

Cell line	Days PIS to nuclear transfer*	Nuclear transfer clones made†	Cleavage (%)	Blastocysts, *n*‡	Blastocysts, % of clones§	Blastocysts, % of cleavage
1	35	24	14/24 (58)	6	25	43
2	42	32	20/32 (63)	4	12.5	20
3		33	21/33 (64)	4	12	19
4	17	92	71/92 (77)	19	21	27
5	71	22	18/22 (82)	4	18	22
6	101	22	15/22 (61)	4	18	27
7	6	44	36/44 (82)	9	20	25
7	6	33	2/33 (6)	0	0	0
8	13	57	43/57 (75)	11	19	26
9	20	42	20/42 (48)	6	14	30
10	27	74	61/74 (82)	12	16	20
11	14	23	17/23 (74)	4	17	24
11	14	5	4/5 (80)	2	40	50
12	21	47	40/47 (85)	4	8.5	10
13		28	21/28 (75)	6	21	29
14¶	54	39	21/39 (54)	6	15	29
15¶	61	42	36/42 (86)	8	19	22
	Total	659	460/659 (70)	109	15%	24%

*PIS, postimmunosurgery. Trophoblast cells were removed and culture of ICM cells was initiated.
†Each clone is the product of attempted fusion of an ICM cell with an enucleated oocyte.
‡Number of blastocysts after *in vitro* culture of the clones for 7 days.
§Frequency of clones becoming blastocysts after 7 days of culture.
¶All cell lines were derived from the pooled ICM of 3 blastocysts, except lines 14 and 15, each of which was derived from the ICM of a single blastocyst.

Table 3. Effect of sire on efficiency of stem cell line production and frequency of blastocysts derived from fusion of stem cells into enucleated oocytes [nuclear transfer (NT)]

	Sire breed	NT clones made, *n*	NT clones becoming blastocysts	
			n	%
A	Angus	89*	14	15.7
B	Holstein	114*	23	20.2
C	Holstein	272*	42	15.4
D	Brahman	184*	30	16.3
E	Brahman	46†	0	0
F	Longhorn	93†	14	15
G	Holstein	102†	22	22
H	Holstein	60†	6	10
I	Holstein	88†	19	22

*Stem cells were fused into enucleated oocytes by using PEG.
†Stem cells were fused into enucleated oocytes by electrofusion.

appears to be partially dependent on the genetics of the embryo as indicated by differences among sires in the frequency of stem cell line formation and blastocyst formation (Table 3).

Totipotency of cultured ICM cells from five cultured cell lines was determined by transfer into cows of blastocysts derived from ICM cell nuclear transfer (Table 4).

Thirty-four of 42 blastocysts derived from cell lines cultured for 6, 13, 20, 27, or 101 days were transferred into uteri of 27 cows. Thirteen of the cows (49%) became pregnant. At 180 days of gestation, 5 (19%) were still carrying 5 (15%) fetuses with heart beats clearly imaged with ultrasonography. Four of the cows delivered normal calves derived from the cultured ICM cells after gestations of normal length. The birth weights of the calves were 75, 80, and 85 pounds for 3 female calves and 86 pounds for a male calf. The gestations were 279, 280, 280, and 279 days, respectively.

The cell cultures producing offspring were cultures 7, 9, and 10. Cell culture 7 was derived from embryos sired by Holstein bull 9805 and the calf born from this culture was Holstein. Cell culture 9 was derived from embryos sired by Brahman bull 9813 and the two calves born were half Brahman. Cell culture 10 was derived from embryos sired by Longhorn bull 12199 and the calf was half Longhorn. DNA typing by Marijo Kent (31) established that each calf was sired by the sire producing the ICM cells from which the calf was derived. The calves were karyotyped and two half-sister Brangus calves from cell line 9 showed tetraploidy of <10% in some lymphocytes at birth but lost the tetraploid lineage by 1 year of age. Karyotypes of integument fibroblasts were normal.

DISCUSSION

The ICM cell culture system reported here prevents differentiation by culturing cells as a loose suspension with <1500 cells per 10-μl drop. Without cell–cell contact, neither cell aggregation nor monolayer formation occurred.

The results presented in Table 3 show that at least some of the ICM cells retain totipotency after culture. The efficiency of blastocyst production from use of the cultured ICM cells in nuclear transfer (15% or 25% of cleaved) is similar to the efficiency of using morulae cells as the donated nucleus in conventional nuclear transfer (18%; ref. 32).

The frequencies of pregnancies (49%) and 180-day maintained pregnancies (19%) after transfer into cows of embryos derived from cultured ICM cells were also similar to the frequency of pregnancies (30%) or maintained pregnancies reported for conventional bovine nuclear transfer (32, 33). The frequency of transferred blastocysts resulting in born offspring was also similar to bovine nuclear transfer and the calves were from three different cell lines.

Pluripotency has been demonstrated previously for cultured cattle ICM cells (12, 18, 20, 21, 27). Our present research was reported as an abstract in 1993 (34). This work demonstrates totipotency from cultured ICM cells of domestic animals, as evidenced by offspring, and the successful use of cultured cells for nuclear transfer (for review of other species, see ref. 12). The methods presented here allowed establishment of ICM cell lines from ≈70% of the blastocysts attempted, when the line was formed from a pool of 3 ICMs. This is approximately equal to the best efficiencies in the production of mouse ES cell lines (6). In mice, some ES cell lines have been shown to be of abnormal karyotype, particularly after several passages. Whether the tetraploidy of calves from line 9 was due to nuclear transfer or ICM cell culture is unknown. While the efficiency of fusion was acceptable in this study (68%), manufacturers' lots of PEG vary considerably in fusogenic activity. A modified electrofusion procedure was used with success for production of blastocysts from cells derived from sires E, F, G, H, and I in Table 3.

The greatest need will be for cell culture systems that promote much greater mitotic activity than the present system while inhibiting differentiation. The CR1aa/SIT/5% FCS culture medium used for this study is adequate only for short-term culture and represents a mere beginning in identification of an optimal culture system for bovine ICM cells.

With the development of culture systems allowing a high rate of cell multiplication, bovine ES cells derived from ICMs or earlier embryo stages should prove useful in propagation and genetic modification of cattle. The use of ES cells in gene transfer could provide more efficient gene transfer with opportunities to select cells for gene integration or expression before offspring are made and opportunities through homol-

Table 4. Production of calves from blastocysts derived from fusion of cultured ICM cells into enucleated bovine oocytes

Cell line*	Days PIS to NT†	NT clones made, *n*	Blastocysts from NT,‡ *n*	Blastocysts (*n*) transferred into cows (*n*)	Cows pregnant at 42 days gestation, *n*	Blastocysts surviving as fetuses *in utero* at gestation day				Calves born
						56	70	150	180	
7	6	44	9	9 into 6	4	5	4	2	2	1
8	13	57	11	6 into 4	0	—	—	—	—	—
9	20	42	6	6 into 4	3	4	4	4	2	2
10	27	74	12	9 into 9	3	1	1	1	1	1
6	101	22	4	4 into 4	3	0	—	—	—	—
			Total	34 into 27	13 of 27 (49%)	10	9 (27%)	7 (21%)	5 (15%)	4 (12%)

*Each of these cell lines was established from the pooled ICMs of 3 blastocysts.
†PIS, postimmunosurgery and start of ICM cell cultures.
‡Number of blastocysts developed per number of clones made after 7 days of culture.

ogous DNA recombination to be site specific in gene transfer or deletion. Bovine ES cells when used as nuclear donors in nuclear transfer could allow the production of large numbers of clonal offspring from one valuable embryo or from the genetically modified ES cells of one valuable embryo.

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Influence of combined activation treatments on the success of bovine nuclear transfer using young or aged oocytes

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Abstract

This study was determined if the combined activation of young or aged oocytes influence their development. The 16-cell stage in vitro maturation/fertilization embryos were exposed to 10 μM nocodazole for 18–20 h, blastomeres that divided within 3 h after the release from nocodazole were used as synchronized donor blastomeres. Metaphase II oocytes were enucleated at 20–22 h post onset of maturation. Enucleated oocytes were divided into 2 groups: oocytes activated at 24 h (young) and oocytes activated at 38 h (aged). In both groups (young and aged), one group of oocytes was activated in 7% ethanol alone for 5 min (alone) and the other group (combination) was activated in ethanol and subsequently incubated in 5 μg/ml cycloheximide in TCM199 for 6 h (combination). Electrofusion was carried out at 30 h (young) and 44 h (aged). The nuclear morphology of the blastomere–oocyte complexes at 1 h post-fusion and their development to the blastocyst stage after 6 days of culture in modified synthetic oviduct fluid were examined. Interphase and swollen nuclei were observed at 1 h post-fusion following nuclear transfer to the cytoplasm from young oocytes of combined activation and aged oocytes of combined and ethanol alone activation. When young oocytes were treated with the combined activation method, the reconstituted embryos had a significantly higher developmental rate to the blastocyst stage than the aged oocyte groups ($P < 0.05$). We conclude that the combined activation of young oocytes leads to a more efficient development of bovine nuclear transfer embryos. © 1997 Elsevier Science B.V.

Keywords: Cattle; Combined activation: Cycloheximide; Nuclear transfer; Young oocyte

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1. Introduction

Smith et al. (1988) suggested that cell cycle synchronous transplantation between nuclei and cytoplasm is important for the development of reconstituted mouse embryos. One of reconstituted mouse embryos. One of the factors which affects the development of reconstituted embryos is the cell cycle stage of nuclear donors (Collas et al., 1992; Cheong et al., 1991, 1993; Campbell et al., 1993; Stice et al., 1993; Techakumphu et al., 1993; De La Fuente and King, 1994; Otaegui et al., 1994; Tanaka et al., 1995a,b).

However, the developmental rates of the blastocyst stage of reconstituted embryos improve with the use of activated recipient oocytes (Stice et al., 1993; Ushijima and Eto, 1993; Aoyagi et al., 1994; Kono et al., 1994). Activation response in bovine oocytes by several activation agents has been demonstrated to be oocyte age dependent. Namely, the development to the pronuclear stage was investigated following activation treatment by ethanol (Nagai, 1987; Yang et al., 1993), calcium ionophore (Ware et al., 1989; Shi et al., 1993), electric pulse (First et al., 1992; Leibfried-Rutledge et al., 1992; Ware et al., 1989; Yang et al., 1993) or cycloheximide (First et al., 1992; Shi et al., 1993; Yang et al., 1993). While less than 40% of the young (23–42 h) IVM oocytes were activated (Nagai, 1987, 1992; First et al., 1992). The relation between activation and aging of recipient oocytes is a factor which affects the development of reconstituted embryos. Consequently, nuclear transfer procedures (routine work) have used aged oocytes (Barnes et al., 1993; Bondioli et al., 1990; Campbell et al., 1993; Keefer et al., 1994; Kono et al., 1994; Sims and First, 1993; Stice and Keefer, 1993; Takano et al., 1993; Willadsen et al., 1991). Mammalian oocyte aging decreases the fertilization rate and the subsequent development (Maurer and Foote, 1971; Seidel et al., 1976). Therefore, using young recipient oocytes is attractive and may increase the overall efficiency of cloning procedures if the oocytes can be activated adequately. Aoyagi et al. (1994) demonstrated that, reconstituted embryos had a high developmental rate to the blastocyst stage when a combination treatment of Ca-ionophore, electric pulse and cycloheximide was used to activate young oocytes. combined ethanol and cycloheximide treatment has been reported to effectively (over 90%) activate freshly matured bovine oocytes (Presicce and Yang, 1994a; Yang et al., 1993). An activation rate of over 80% was observed when pig oocytes were treated with ethanol and were subsequently cultured with cytoheximide (Petr et al., 1996). This study examined the influence on the development rates of the combined activation exposure to 7% ethanol and 5 μg/ml cycloheximide of young (24 h) and aged (38 h post-onset maturation) oocytes, when nuclear donors were used blastomeres synchronized.

2. Materials and methods

2.1. Nuclear donor embryos

Bovine ovaries were collected from a slaughterhouse and were brought to the laboratory within 4 h after slaughter. Oocytes were collected by aspiration of the antral

follicles (2–7 mm diameter) with an 18-gauge needle and a sterile syringe. Cumulus–oocyte complexes (COCs) were washed twice with modified tyrode's solution (TALP; Bavister et al., 1983). For oocyte maturation, the COCs were cultured in tissue culture medium 199 (TCM-199) supplemented with 25 mM HEPES (Gibco Laboratories, Grand Island, NY, USA), 10% fetal calf serum (FCS; Gibco), 0.02 U/ml FSH, 1 μg/ml estradiol, 21.8 μg/ml sodium pyruvate and 50 μg/ml gentamicin sulfate (Sigma Chemical, St. Louis, MO, USA) for 22 h at 39°C in an atmosphere of 5% CO_2 in humidified air.

Frozen–thawed semen from one Holstein bull was used for in vitro fertilization. Frozen semen was thawed in a water bath for 1 min at 35°C. The motile sperm were separated using 45 and 90% Percoll (Pharmacia BioProcess, Uppsala, Sweden) diluted with an isotonic medium (B.O.; Brackett and Oliphant, 1975) by centrifugation at $700 \times g$ for 20 min. The sperm were then washed using B.O. medium without bovine serum albumin (BSA; Sigma) by centrifugation at $500 \times g$ for 5 min. The sperm were co-incubated with about 15 COCs at the concentration of 5×106 cells/ml in a 100 μl microdrop of B.O. medium containing 3 mg/ml BSA and 2.5 mM theophylline (Sigma), covered with paraffin oil for 18 h at 39°C under 5% CO_2 in air (Takahashi and First, 1993).

After insemination, the cumulus cells were removed from the oocytes by vortex agitation in TALP. Cumulus-free oocytes were cultured in 50 μl microdrops of modified synthetic oviduct fluid medium (mSOF; Takahashi and First, 1992) supplemented with 3 mg/ml BSA, 20 amino acids and 10 μg/ml insulin (Sigma) for 81 h at 39°C under 5% CO_2, 5% O_2 and 90% N_2. The 16-cell stage embryos at 99 h post-insemination (hpi) were selected to prepare donor nuclei for nuclear transfer.

2.2. Recipient cytoplasm

The COCs collected from ovaries were cultured in the same maturation medium. At 20–22 h post-maturation, the cumulus cells were removed and the oocytes with first polar bodies were selected as recipients. The oocytes were then placed in microdrops of Dulbecco's phosphate-buffered saline (DPBS; Whittingham, 1971) containing 20% FCS, 5 μg/ ml cytochalasin B (Sigma) and 0.3 μg/ml nocodazole (Aldrich Chemical, Milwaukee, WI, USA), and enucleated by removing the first polar body and the adjacent cytoplasm presumably containing the nuclear material (Prather et al., 1987). Enucleation was confirmed by staining the oocytes with 5 μg/ml Hoechst 33342 (Sigma) (Westhusin et al., 1990). The enucleated oocytes were divided into two groups: oocytes activated at 24 h (young) and oocytes activated at 38 h (aged). The oocytes were divided into two groups (young or aged): (1) One group of oocytes was activated in 7% ethanol in TCM-199 supplemented with 24 mM HEPES, 10% FCS, 0.2 mM sodium pyruvate and 50 μg/ml gentamicin for 5 min (alone) and then was placed in the maturation medium for 6 h. (2) The other group (combination) was activated in ethanol and then was incubated in 5 μg/ml cycloheximide in TCM-199 for 6 h (Presicce and Yang, 1994b).

2.3. Nuclear transfer and membrane fusion

The zonae pellucidae of the 16-cell stage donor embryos were removed by incubation in 0.5% pronase (Actinase E, Kaken Pharmaceutical, Tokyo, Japan) dissolved in DPBS. Blastomeres of the donor embryos were isolated by pipetting the embryos in Ca^{2+}–Mg^{2+}-free DPBS. Nuclear transplantation was carried out as described by McGrath and Solter, 1983, 1984). A blastomere of the donor embryo was introduced into the perivitelline space of the enucleated oocyte at 30 h (young) and 44 h (aged oocytes) post-maturation using an injection pipette with an inner diameter of 35–40 μm through a slit in the zona.

The blastomere–oocyte complexes were placed in an electrode chamber (BTX, San Diego, CA, USA) filled with 0.3 M mannitol solution containing 0.1 mM $MgSO_4$, 0.05 mM CaCl2 and 0.05 mg/ml BSA (Cheong et al., 1993). They were aligned by exposure to alternating current (AC) pulses of 0.6 MHz, 10 V for 6 s. Thereafter, two direct current (DC) pulses of 1.0 kV/cm for 70 μs (each pulse 1 s apart) were applied to fuse the membrane of the blastomere and enucleated oocyte (Cheong et al., 1991) using an Electro Cell Fusion instrument (LF 100, Life Tec., Tokyo, Japan).

2.4. In vitro culture of nuclear transfer embryos

About 10 blastomere–oocyte complexes were cultured in a 50 μl drop of mSOF supplemented with 3 mg/ml BSA and 10 μg/ml insulin for 6 days (147 h post-fusion) at 39°C under 5% CO_2, 5% O_2 and 90% N_2.

2.5. Experiment 1

This experiment determined if the combined activation of young or aged oocytes influence the nuclear morphology at 1 h post-fusion. Resumption of cleavage was induced in the donor embryos by the method of Tanaka et al. (1995a). In brief, the 16-cell stage embryos at 99 hpi were incubated in mSOF supplemented with 10 μM nocodazole (Aldrich Chem) for 18–20 h at 39°C under 5% CO_2, 5% O_2 and 90% N_2 and it was presumed that synchronous cleavage of donor cell was induced (Fig. 1). The zonae pellucidae were then removed by incubation with 0.5% pronase in DPBS. Blastomeres were isolated by pipetting the embryos in Ca^{2+} and Mg^{2+}-free DPBS. Blastomeres were isolated by pipetting the embryos in Ca^{2+} and Mg^{2+}-free DPBS. Isolated blastomeres were incubated in mSOF without nocodazole in multi-well plates (Sumitomo Bakelite, Tokyo, Japan) for 3 h at 39°C under 5% CO_2, 5% O_2 and 90% N_2. The blastomeres that divided within 3 h after release from nocodazole were used as synchronized donor blastomeres (3–6 h post-division).

The blastomeres used as nuclear donors were fused with four groups of activated enucleated oocytes (young or aged, and alone or combined activation treatments, respectively). At 1 h after fusion, the reconstituted embryos were mounted on a slide and in a vaseline–paraffin mixture under a coverslip. The slides were immersed in ethanol:acetic acid (3:1) for one night and the reconstituted embryos were stained with 1% aceto-orcein. The slides were decolored in glycerol:acetic acid:distilled water (1:1:3)

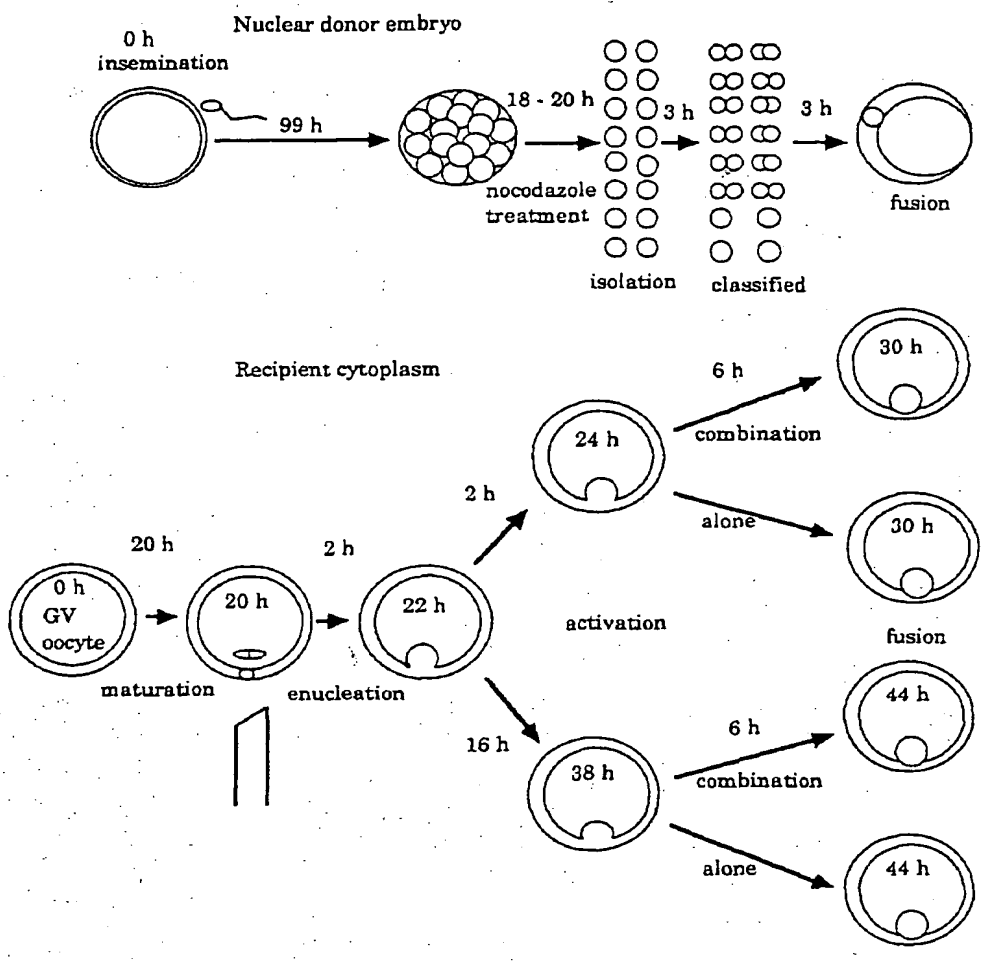

Fig. 1. Nuclear transfer methods.

(decolor solution). Nuclear morphology was examined under a phase-contrast microscope.

2.6. Experiment 2

This experiment determined if the combined activation of young or aged oocytes influenced bovine nuclear transfer embryonic development. The reconstituted embryos were produced as in experiment 1. The reconstituted embryos were cultured for six days, and the fusion rates of the blastomeres and oocytes and the cleavage rates of the reconstituted embryos were investigated at 2 and 48 h post-fusion, respectively. The nuclear transfer embryos were examined for development to the blastocyst stage under a stereomicroscope. The cells of embryos that developed to blastocysts were counted (Takahashi and First, 1992).

Table 1
Influence of the combined activation of young or aged oocytes on nuclear morphology at 1 h post fusion

Recipient cytoplasm	Activation	No. oocytes used	Fused (%)	No. of nuclear features		
				PCC[a]	Mitotic phase	Swollen nucleus
Young oocyte	Combination	20	90.0	0	0	18
	Alone	25	84.0	12 (57.1%)	9 (42.9%)	0
Aged oocyte	Combination	23	91.3	0	0	21
	Alone	22	90.9	0	0	20

[a]Premature chromosome condensation.

2.7. Statistical analysis

In experiment 2, the results were obtained from five replicates and the data were analyzed by Two-way ANOVA and Fisher PLSD.

3. Results

3.1. Experiment 1

Following the transfer of the nucleus to the recipient cytoplasm after the combined activation of young and aged oocytes, and another sample of aged oocytes activated by ethanol alone, resulted in swollen interphase nuclei in all cases at 1 h post fusion. When young oocytes activated in 7% ethanol alone were used as the recipient cytoplasm, premature chromosome condensation (PCC) and the mitotic phase were observed at a rate of 57.1 and 42.9%, respectively (Table 1).

3.2. Experiment 2

When young oocytes activated in 7% ethanol alone were used as recipient oocytes, blastomere–oocyte complexes had a significantly lower fusion rate compared to the other three groups ($P < 0.01$) (Table 2). The reconstituted embryos produced by young oocytes activated in ethanol alone had a significantly lower cleavage and developmental

Table 2
Influence of the combined activation of young or aged oocytes on the developmental rate of bovine nuclear transfer embryos[a]

Recipient cytoplasm	Activation	No. oocytes used	Fused %	Cleaved[b] %	Blastocytes[b] %	Cell no. in blastocyst
Young oocyte	Combination	112	92.5 ± 2.3^{c}	88.8 ± 1.4^{ef}	42.7 ± 1.4^{e}	110.5 ± 8.6
	Alone	75	78.8 ± 3.6^{d}	78.0 ± 6.6^{e}	13.7 ± 4.6^{f}	128.3 ± 18.3
Aged oocyte	Combination	87	92.3 ± 1.6^{c}	97.0 ± 2.0^{f}	29.0 ± 4.3^{g}	94.0 ± 8.5
	Alone	112	94.3 ± 2.4^{c}	96.0 ± 1.1^{f}	28.0 ± 2.1^{g}	89.1 ± 6.9

[a]Results were mean ± s.e.m. from five replicates.
[b]Percentage of cleaved oocytes and blastocysts were calculated from the number of fused embryos.
[c,d,e,f,g]Values with different superscripts differ significantly ($^{cd}P < 0.01$, $^{efg}P < 0.05$).

rates to the blastocyst stage than the two groups (combination and ethanol alone) of aged oocytes ($P < 0.05$). The highest developmental rate to the blastocyst stage was observed in the combined activation group of young oocytes ($P < 0.05$). A statistical difference was not observed between ethanol alone and the combined activation groups of aged oocytes. The cell numbers of the reconstituted embryos that developed to the blastocyst stage showed no difference among the four groups.

4. Discussion

Oocytes in metaphase II (M-II) have high levels of maturation promoting factor (MPF) (Barnes et al., 1993; Campbell et al., 1993). Concluding that the reconstituted embryos do not have the ability to maintain correct ploidy in the nuclei and normal development cannot be expected when nuclear donors other than G1-phase nuclei are transferred into enucleated M-II oocytes seems reasonable (Johnson and Rao, 1970; Campbell et al., 1993). However, if M-II oocytes undergo optimum activation, the level of MPF decreases and disappears in the oocytes (Barnes et al., 1993; Campbell et al., 1993). When nuclear donors at interphase are transferred into the oocytes with lower levels of MPF activity, the reconstituted embryos maintain correct ploidy and normal development is expected (Barnes et al., 1993; Campbell et al., 1993). Therefore, if M-II oocytes undergo optimum activation, the reconstituted embryos must have a higher developmental rate than untreated M-II oocytes. Previous reports have confirmed that the activation of recipient cytoplasm is important for the development of reconstituted embryos (Stice et al., 1993; Aoyagi et al., 1994; Kono et al., 1994; Campbell et al., 1994).

It has been hypothesized that matured mammalian oocytes constantly synthesize groups of highly transient, liable proteins, such as the cytostatic factor (CSF) or the c-mos proteins, which maintain the function and the persistent high level of MPF (Parrish et al., 1992). The fertilizing sperm normally initiates numerous, periodic elevations of intracellular free calcium in oocytes over several hours depending on the species (Cuthberston et al., 1981; Miyazaki, 1990; Kline and Kline, 1992; Fissore and Robl, 1992; Vitullo and Ozil, 1992). The function of these Ca^{2+} elevations is believed to destroy the existing and the nascent CSF, and the cause inactivation of MPF and resumption of meiosis (Parrish et al., 1992). Several Ca^{2+}-elevating agents, including ethanol, have been used to induce oocyte activation in a number of species. It is believed that a single Ca^{2+} elevation induced by the single artificial stimulus (ethanol or electric pulse) can destroy only the existing CSF and would not prevent the renewal of CSF (Cuthberston et al., 1981; Fissore and Robl, 1992). The CSF is believed to be continuously synthesized in the young, but not in aging oocytes; only aged oocytes can be readily activated by a single Ca^{2+} elevation stimulation (Presicce and Yang, 1994a). When a single electric pulse was given to oocytes at 29 h of maturation (young oocytes), the MPF decreased initially, but recovered to its full activity a few hours later (Collas et al., 1993). When recipient oocytes with a high level of MPF were used in the nuclear transfer, the nuclear features PCC and mitotic phase were observed after 1 h post-fusion.

However, when they were used after the inactivation of MPF, only the interphase nuclei (swollen nucleus) could be seen (Campbell et al., 1993; Sotomaru et al., 1994).

The results of experiment 1, showed that when young oocytes were activated with ethanol alone, the nuclear features observed were PCC and mitotic phase after 1 h post-fusion. When combined activation was used on the young oocytes, only the interphase (swollen nucleus) was observed. Therefore, this result may lead to the conclusion that CSF decreases transiently and recovers to its full activity in young oocytes activated with ethanol alone after 6 h post-activation and that the MPF level in the oocyte is high at the time of fusion.

The results of experiment 2 showed that the difference in the activation methods do not affect the development of reconstituted embryos produced by aged oocytes, and that combined activation improves the development of reconstituted embryos produced by young oocytes. Lavoir et al. (1996) reported that the combined activation (ionomycin and 6-dimethylaminopurine) for young M-II oocytes improves the developmental rate to the blastocyst stage in nuclear transfer. When activation with ethanol alone was used on young oocytes, development of the reconstituted embryos to the blastocyst stage could not be improved. However, the subsequent cycloheximide exposure would prevent the renewal of CSF synthesis and the inactivation of MPF would be maintained in the young oocytes by combined activation. The MPF in the oocytes is low at the time of fusion and the developmental rate could be improved.

In spite of the activation of aged oocytes, only interphase nuclei (swollen nucleus) were observed in all cases at 1 h post-fusion, which suggests that MPF in aged oocytes maintain inactivation until fusion time. When the young oocytes activated by the combined method were used as recipient oocytes, development to the blastocyst stage was improved at a higher rate compared to aged oocytes. Cytoskeletal deterioration is well known to occur more often in aged mouse oocytes than in young mouse oocytes (Webb et al., 1986), and aged bovine oocytes are well known to reduce fertilization and developmental rates. This study showed that the development of the reconstituted embryos is negatively affected by using aged oocytes as recipient cytoplasm on bovine nuclear transfer.

In conclusion, this study confirmed that the combined activation (7% ethanol and 5 μg/ml cycloheximide) of young oocytes leads to a more efficient development of bovine nuclear transfer embryos.

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Human Factor IX Transgenic Sheep Produced by Transfer of Nuclei from Transfected Fetal Fibroblasts

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Ovine primary fetal fibroblasts were cotransfected with a neomycin resistance marker gene (*neo*) and a human coagulation factor IX genomic construct designed for expression of the encoded protein in sheep milk. Two cloned transfectants and a population of neomycin (G418)–resistant cells were used as donors for nuclear transfer to enucleated oocytes. Six transgenic lambs were liveborn: Three produced from cloned cells contained factor IX and *neo* transgenes, whereas three produced from the uncloned population contained the marker gene only. Somatic cells can therefore be subjected to genetic manipulation in vitro and produce viable animals by nuclear transfer. Production of transgenic sheep by nuclear transfer requires fewer than half the animals needed for pronuclear microinjection.

Microinjection of DNA into the pronuclei of fertilized oocytes has been the only practical means of producing transgenic livestock since the method was established in 1985 (*1*). However, only a small proportion (~5%) of animals integrate the transgene DNA into their genome (*2*, *3*). In addition, because the timing and site of integration are random, many transgenic lines do not provide sufficiently high levels of transgene expression or germline transmission. The consequent inefficient use of animals and associated high costs are a major drawback to pronuclear microinjection.

In mice, embryonic stem cells provide an alternative to pronuclear microinjection as a means of transferring exogenous DNA to the germline of an animal and allow precise genetic modifications by gene targeting (*4*, *5*). However, despite considerable efforts, embryonic stem cells capable of contributing to the germline of any livestock species have not been isolated (*6*–*11*).

Recently, viable sheep have been produced by transfer of nuclei from a variety of somatic cell types cultured in vitro (*12*–*14*). We now demonstrate that nuclear transfer from stably transfected somatic cells provides a cell-mediated method for producing transgenic livestock.

We have used a transgene designed to express human clotting factor IX (FIX) protein in the milk of sheep. FIX plays an essential role in blood coagulation, and its deficiency results in hemophilia B (*15*). This disease is currently treated with FIX derived mainly from human plasma. Recombinant FIX produced in milk would provide an alternative source at lower cost and free of the potential infectious risks associated with products derived from human blood.

The transgene construct, pMIX1 (*16*), comprises the human FIX gene, containing the entire coding region (*17*), linked to the ovine β-lactoglobulin (BLG) gene promoter, which has been previously shown to provide a high level of transgene expression in ovine mammary glands (*18*). Analysis of pMIX1 expression in transgenic mice showed that seven of seven female founders expressed FIX in their milk (*19*). The level of expression in two animals (125 μg/ml) exceeded that achieved in previous studies (*20*, *21*), indicating that pMIX1 is functional and suitable for introduction into sheep.

Primary strains of ovine cells, termed PDFF (Poll Dorset fetal fibroblast) 1 to 7, were derived from seven day-35 fetuses from the specific pathogen-free flock at PPL Therapeutics (*22*). Sex analysis of each cell strain by the polymerase chain reaction (PCR) (*23*) revealed PDFF5 to be male and

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Table 1. Results of nuclear transfer. Nuclear transfer was performed as described previously (*12, 13*). All cells were exposed to a reduced serum concentration (0.5%) for 5 days before use as nuclear donors. PDFF5 cells were used for nuclear transfer at passage 2 or 3, PDFF2 transfected pools at passage 5 to 7, and transfected clones PDFF2-12 and PDFF-13 at passage 7 to 9. Liveborn lambs were defined as those with a heartbeat and able to breathe unassisted at birth.

Measurement	PDFF5 (non-transfected)	PDFF2 pool	PDFF2-12	PDFF2-13
Reconstructed embryos	82	224	89	112
No. developed to morulae or blastocysts	5 (6.1%)	22 (9.8%)	19 (21.4%)	23 (20.5%)
Embryos transferred	5	22	19	21
Recipients	2	9	7	6
Pregnancies at day 60	2	4	4	1
Fetuses at day 60 (% of embryos transferred)	3 (60%)	4 (18.2%)	6 (31.6%)	1 (4.8%)
Liveborn lambs (% of embryos transferred)	1 (20%)	3 (13.6%)	2 (10.5%)	1 (4.8%)
Nuclear transfer efficiency (% live lambs from reconstructed embryos)	1.22%	1.34%	2.25%	0.89%

the other six to be female.

Trial experiments indicated that both PDFF2 and PDFF5 cells could be readily transfected with a *lacZ* reporter gene with the use of the cationic lipid reagent Lipofectamine. PDFF2 cells at passage 1, after 3 days in culture, were cotransfected with pMIX1 DNA and the selectable marker construct PGKneo, and stable transfectants were selected with G418. Because the effects of drug selection and growth as single-cell clones on the ability of cells to support nuclear transfer were unknown, cells were then treated in two ways: One group was grown at high density under G418 selection and then cryopreserved as a pool for nuclear transfer. The other group was plated at low density under G418 selection, and cloned transfectants were grown from isolated colonies (*22*). A total of 24 clones was isolated, of which 21 were expanded for analysis of genomic DNA. Ten clones were found to contain pMIX1 by DNA hybridization analysis (*24*).

Untransfected PDFF2 cells cultured to passage 19 over a period of 80 days exhibited a modal chromosome number of 54, the euploid ovine chromosomal complement. The chromosome number of the four most rapidly growing pMIX1-transfected clones (PDFF2-12, -13, -31, -38) was determined at passage 6 or 7, after an average of 40 days in culture, and that of the uncloned PDFF2 pool was determined at passage 5, after 19 days in culture. Each clone and the pool showed a modal chromosome number of 54, indicating the absence of gross chromosomal instability during culture and drug selection.

We have proposed that induction of quiescence in nuclear donor cells by serum deprivation is necessary for successful nuclear transfer (*12*). After 5 days of culture in medium with a reduced serum content (0.5%), immunofluorescence detection of proliferating-cell nuclear antigen (PCNA), which is an indicator of active DNA replication, showed that none of the cells analyzed was in S phase, consistent with cell cycle arrest (*25*). Restoration of serum content to 10% reversed this effect and cell growth resumed.

Four cell types were used as nuclear donors: untransfected male PDFF5 cells, pooled female PDFF2 transfectants, and two transfected clones, PDFF2-12 and PDFF2-13, which contained >10 and ~5 copies of the pMIX1 transgene, respectively. Transfer of nuclei from each cell type into enucleated oocytes derived from Scottish Blackface ewes was performed as previously described (*12, 13*).

Live lambs were obtained from all four cell types (Table 1). As expected, animals derived from PDFF5 cells were male and those from PDFF2 cells were female. The efficiency of nuclear transfer, expressed as the number of liveborn lambs obtained per 100 reconstructed embryos, varied from 0.89% for PDFF2-13 to 2.25% for PDFF2-12. This efficiency is similar to the value (1.35%) that we obtained previously for nonmanipulated fetal fibroblasts from another breed of sheep (BLWF1) (*13*).

Pregnancies resulting from embryo trans-

Table 2. Characteristics of nuclear transfer–derived lambs. Outcomes of 11 pregnancies resulting from nuclear transfer of PDFF donor cells. When judged necessary, labor was induced by injection of dexamethasone at day 153 of gestation; when required, cesarean section (CS) was performed 24 to 52 hours later. The average duration of gestation for the Poll Dorset flock at PPL Therapeutics is 145 days.

Pregnancy no.	Nuclear transfer donor cell type	Lamb	Gestation (days)	Birth weight (kg)	*neo*	FIX	Sex	Comments
1	PDFF5	7LL5	147	3.8			M	Unassisted birth
2 (twins)	PDFF5	7LL6*	150	3.4			M	Stillbirth, one fetus dead for ≤1 week
		7LL7*	150	3.7			M	
3	PDFF2 pool		<80					Regressed
4	PDFF2 pool	7LL8	155	7.6	(+)	(−)	F	Assisted birth because of position of lamb
5	PDFF2 pool	7LL9*	161	6.3	(+)	(−)	F	Induced, CS 52 hours later, died 90 min postpartum, meconium in lung
6	PDFF2 pool	7LL12	155	8.7	(+)	(−)	F	Induced, CS 52 hours later
7	PDFF2-12	7LL3*	130				F	Spontaneous abortion
8 (twins)	PDFF2-12	7LL10*	132	3.6	(+)	(+)	F	Loss of fetal heartbeat, induced, CS,
		7LL11*	132	4.5	(+)	(+)	F	stillbirth, one fetus abnormal
9	PDFF2-12	7LL14*	148	3.6	(+)	(+)	F	Induced, CS 24 hours later, heartbeat, no breathing
10 (twins)	PDFF2-12	7LL15	155	4.6	(+)	(+)	F	Induced, unassisted birth, 7LL16
		7LL16*	155	3.0	(+)	(+)	F	euthanized at 14 days, heart defect
11	PDFF2-13	7LL13	155	5.5	(+)	(+)	F	Induced, unassisted birth

*Lamb died or was euthanized for animal welfare reasons.

fer were determined by ultrasound scan at about 60 days after estrus, and development was subsequently monitored at regular intervals. Of the original 14 fetuses, 7 were liveborn, as defined by heartbeat and unassisted breathing (Table 2). Postmortem examination of aborted fetuses and dead lambs did not reveal any common factor as a cause of death.

All animals derived from PDFF cells exhibited a prolonged gestation, and, with the exception of animals 7LL5 to 7LL8, labor was induced artificially. Delayed delivery was likely the cause of death of lamb 7LL9. Subsequently, all surrogate ewes were induced at day 153, and, if necessary, cesarean section was performed. Three of 11 pregnancies were twin pregnancies. In two instances (7LL6 and 7LL7 and 7LL10 and 7LL11), the death of one fetus in late pregnancy probably resulted in the death of the sibling.

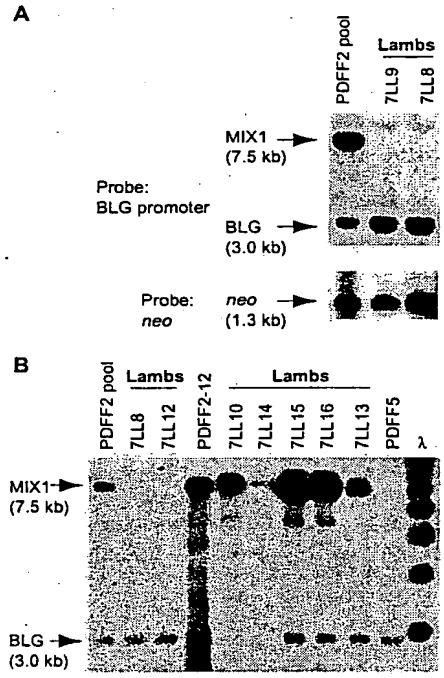

Fig. 1. DNA analysis of transfected clones and transgenic sheep. Genomic DNA was isolated from the blood of live animals or tongue samples from dead animals, digested with Bam HI and Eco RI, and subjected to Southern hybridization with either a 1.8-kb fragment of the BLG promoter or the *neo* gene. (**A**) Southern analysis of the uncloned pool of cells (PDFF2 pool), and two lambs (7LL8 and 7LL9) derived from them, for the presence of pMIX1 and PGKneo. (**B**) Assay for the presence of the pMIX1 transgene in lambs derived from the PDFF2 pool (7LL8 and 7LL12) and from the transfected clones PDFF2-12 (7LL10, 7LL14 to 7LL16) and PDFF2-13 (7LL13). PDFF5 cells were not transfected. The positions and sizes of fragments corresponding to the transgenes and the endogenous BLG gene are indicated. The lane marked λ is a 1-kb ladder of phage λ fragments from 3 to 12 kb.

The birth weight of nuclear transfer–derived lambs whose gestation exceeded 145 days ranged from 3.0 to 8.7 kg, with a mean of 3.7 kg for twins and 5.9 kg for single pregnancies. Poll Dorset lambs in the PPL Therapeutics New Zealand–derived flock have mean weights of 3.75 kg for twins and 5.1 kg for single pregnancies. However, comparison is complicated by the fact that nuclear transfer–derived lambs were gestated in Scottish Blackface surrogate mothers. All animals from PDFF2 cells had an undershot lower jaw that did not interfere with their well-being. This characteristic is a genetic trait that occurs sporadically in the Poll Dorset breed and is considered to be unrelated to nuclear transfer. The PDFF5 lambs did not show this feature.

DNA from nuclear transfer–derived lambs was analyzed for the presence of pMIX1 and PGKneo transgenes (Fig. 1). All fetuses and animals derived from the transfected PDFF2 cells were transgenic. The three animals derived from the PDFF2 pool (7LL8, -9, -12) contained the selectable marker gene but lacked the FIX transgene (Fig. 1, A and B). Fetuses and lambs derived from the cell clones PDFF2-12 (7LL10, -14, -15, -16) and PDFF2-13 (7LL13) contained both the FIX transgene (Fig. 1B) and PGKneo.

Our approach has shown that cell-mediated transgenesis is possible in a mammal other than the mouse. The technique is still in the early stages of development and problems remain to be addressed—in particular, the lack of spontaneous parturition and the incidence of perinatal mortality. However, the mortality rate we observed (46%) was exacerbated by two twin pregnancies in which the death of one lamb in late gestation may have resulted in the loss of the other. The mortality rate for nontwin pregnancies was 28.6%, higher than that occurring after normal breeding (~8%) but similar to that observed after nuclear transfer with embryonic blastomeres (5 to 40%) (26). Our data therefore do not suggest any correlation between lamb mortality and extended culture or genetic manipulation of the donor cell. Many types of manipulation of preimplantation embryos—for example, in vitro oocyte maturation and fertilization, in vitro culture, asynchronous embryo transfer, and progesterone treatment of the mother—have been shown to increase fetal morbidity and mortality (26, 27). An increased understanding of the interaction between the transplanted nucleus and the host cytoplasm and the relation between the early embryo and the maternal environment, together with improved culture systems, should increase the success of embryo production and manipulation in vitro.

The use of somatic cell donors for nuclear transfer in livestock offers many advantages over pronuclear microinjection. Since 1989, PPL Therapeutics has generated a substantial number of transgenic sheep by pronuclear microinjection. A total of 51.4 animals are required to produce one transgenic lamb by pronuclear microinjection, compared with 20.8 animals in the present study by nuclear transfer, values that differ by a factor of ~2.5 (Table 3). The most important difference is that no recipients are wasted gestating nontransgenic lambs in the nuclear transfer technique.

Gestation of large numbers of nontransgenic embryos represents a major source of inefficiency (28). Several schemes have been devised to identify transgenic embryos before embryo transfer, either by detection of the transgene in embryo biopsies by PCR (29) or by co-expression of a marker gene (30, 31). However, these methods, with the possible exception of that of Takada *et al.* (30), are restricted by the persistence of

Table 3. Comparison of the production of transgenic sheep by nuclear transfer or pronuclear microinjection.

Parameter	Pronuclear microinjection (1989–1996)	Nuclear transfer of PDFF2 transfectants
Oocyte donors	982	68
Intermediate recipients*	Not applicable	14
Final recipients	1895	22
Total number of sheep used	2877	104
Established pregnancies (% of final recipients)	912 (48%)	9 (41%)
Lambs born	1286	6
Viable transgenic lambs born†	56	5
Percentage of offspring transgenic	4.35%	100%
Sheep required for production of one transgenic lamb	51.4	20.8

*After nuclear transfer, intermediate recipients are used to allow development of reconstructed embryos to blastocyst stage. †Defined as those alive at 1 week of age.

unintegrated DNA during the short time that embryos can be cultured before embryo transfer. In contrast, cells transfected in vitro can be analyzed extensively before effort is devoted to large animals. This advantage will be particularly important in instances in which microinjection is inefficient; for example, with large constructs such as yeast artificial chromosomes.

Delayed integration of microinjected DNA into the embryo genome often results in mosaic founder animals. The reduced rate of transgene transmission resulting from germline mosaicism can hinder or prevent the establishment of transgenic lines from potentially valuable founder animals. In contrast, animals produced by nuclear transfer are entirely transgenic.

Nuclear transfer allows the sex of transgenic animals to be predetermined and thus offers a further twofold increase in efficiency relative to pronuclear microinjection when the sex of the transgenic founder animal is critical. If, for example, the primary interest is the expression of human proteins in milk, the founder generation can be all females. Sheep with different random integrations of the transgene can be produced by nuclear transfer from independent cell clones and the milk analyzed. After a suitable clone has been identified, the corresponding stock of cells can be used to generate an "instant flock" by further nuclear transfer. Such a flock could be superior to those produced by conventional breeding as a source of proteins for human therapy because genetic identity would contribute to the consistency of the medicinal product.

The procedures of transfection, drug selection, and growth from single-cell clones described here are essentially the same as those required for gene targeting. The realistic prospect of targeted genetic manipulation in a livestock species should open a vast range of new applications and research possibilities.

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16. For construction of pMIX1, human FIX DNA from an Nhe I site at position 2982 to a Bst BI site at position 33,840 (positions refer to EMBL database file HSFIXG) was derived from overlapping genomic λ clones. A 3′ fragment from the Bst BI site to position 34,346 was derived by PCR amplification and a Xho I site introduced. The 31.36-kb fragment containing the FIX coding region was assembled in Bluescript KS− vector (Stratagene) that had been digested with Xba I and Xho I. The entire FIX gene was then excised as a Not I–Xho I fragment and inserted into the ovine BLG expression vector pMADS+ that had been digested with Not I and Xho I. The pMADS+ vector consists of 4.2 kb of BLG promoter sequence, 30 bp of 5′ untranscribed region, a multicloning site (Eco RV, Not I, Bgl II, Nhe I, Xho I, Cla I), 170 bp of 3′ untranscribed region including a polyadenylate addition site, and 2 kb of 3′ flanking region cloned into pUC18, containing a modified polylinker to allow excision of the insert with either Mlu I or Pvu I.
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19. Transgenic mice were produced by microinjection of a 37.8-kb Mlu I fragment of pMIX1 into the male pronucleus of fertilized mouse oocytes. Milk was collected from transgenic founder females at day 10 postpartum. FIX protein in milk was assayed by enzyme-linked immunosorbent assay with polyclonal rabbit antiserum to human FIX (Dako) and quantified by comparison with purified human FIX standard (Diagnostica Stago).
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22. PDFF cell strains were isolated by disaggregation of day-35 fetuses and cultured as described previously (*13*). At first passage, 2×10^5 PDFF2 cells were plated into a 3.5-cm-diameter well and cotransfected with 0.5 μg of PGKneo and 1.5 μg of Mlu I–digested pMIX1 DNA with the use of Lipofectamine (Gibco). Forty-eight hours after transfection, cells were split 1:10 and G418 added to a final concentration of 0.6 mg/ml. Transfected PDFF2 cells achieved subconfluence after selection for 6 days. At the third passage, one portion of the cells was split 1:10 and then subjected to selection for an additional 5 days before cryopreservation as an uncloned population. Other portions were split 1:1000 or 1:5000 and subjected to G418 selection for an additional 7 days. Individual colonies were isolated and expanded for cryopreservation at passage 5, and a portion of each clone was grown further for chromosome counting and Southern (DNA) blot analysis.
23. The sex of strains PDFF1 to PDFF7 was determined by PCR, essentially as described [R. Griffiths and B. Tiwari, *Mol. Ecol.* **2**, 405 (1993); B. W. Kirkpatrick and R. L. Monson, *J. Reprod. Fert.* **98**, 335 (1993)].
24. Genomic DNA isolated from cloned pMIX1 transfectants was digested with Bam HI and Eco RI and subjected to Southern blot analysis with a 1.8-kb fragment of the BLG promoter.
25. PCNA analysis of serum-deprived cells was performed essentially as described [I. R. Kill *et al.*, *J. Cell Sci.* **100**, 869 (1991)].
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32. We thank J. Bracken and M. Malcolm-Smith for technical assistance in large animal work, H. Bowran and D. McGavin for care of the animals, D. Cottom for providing data on microinjection studies, the Animal Sciences Team at PPL Therapeutics for help in deriving the PDFF cells, Y. Gibson and the Small Animal Unit at PPL Therapeutics for generating FIX transgenic mice, S. Bruce for FIX protein analysis, G. G. Brownlee for human FIX genomic λ clones, D. Melton for PGKneo, A. R. Scott for technical assistance with molecular biology, and I. Garner for constructive discussions during the course of the project. The experiments were conducted under the Animals (Scientific Procedures) Act 1986 and with the approval of the Roslin Institute Animal Welfare and Experiments Committee.

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Dolly Confirmation

It has now been almost a year since the cloning of the sheep Dolly from an adult ovine cell was announced (1). The year has brought much agonizing discussion, potential legislation, and some laurels, but no more Dollies. The principal scientist, Ian Wilmut, has announced (2) that he and his group have no intention of trying again (to clone using mammary DNA and a host denucleated ovine cell). Some "very soon" to be delivered (3) cows that were to be cloned from adult cells have yet to appear. Other rumored events seem also to have dissipated. It is a well-known tenet of science that a single observation is not to be codified until confirmed by someone in some way. The single observation gains some credence when well controlled or of a unique nature, or both. It is the lack of any confirmation that provokes our skepticism; here are some of the detailed reasons.

1. The cloning was done once out of some some 400 tries. Only one successful attempt out of some 400 is an anecdote, not a result. All kinds of imagined and unimagined experimental error can occur.
2. The characterization of the mammary gland cells used as nucleus donors was poor; it could have been one of the donor's rare stem cells that was involved, as acknowledged in the paper (2).
3. The reason why the donor ewe was pregnant was not explained (1). This is important, because the cell which led to Dolly could have been of fetal origin. Why was no analysis of the fetus and its father's genotype performed? Given these DNA fingerprints, or even the sex of the fetus, one could have excluded a fetal cell as donor.
4. The demonstration that the four microsatellite marker DNAs seem the same in Dolly and in the donor mammary cells is good, but not sufficient; it would probably be rejected by a jury called to deliberate on Dolly's origin, not an unlikely event given Dolly's commercial potential. Sheep are highly inbred and there are, to our knowledge, no data on gene frequencies in sheep populations; differences in DNA fingerprints can provide exclusion, but similarities are only a statistic.
5. An analysis of Dolly's mitochondrial DNA has not been given, although it could provide important clues to her origin; the genotype of the recipient oocyte and the mitochondrial genotype of the donor cell or that of any of the other players was also not given.
6. Last summer (4), and again recently (A. E. Schnieke *et al.*, Reports, 19 Dec., p. 2130), the same

group announced the cloning of transgenic sheep, but from a fetal cell not an adult cell; Polly is not a Dolly. Remember, Dolly should be "aged" relative to her peer group. Barring new science, she must have retained any imprinted genes from the previous generation, she should have short telomeres, and her DNA should have an adult's worth of mutations; a special creature in more ways than one.

7. No hint is given in the paper (1) that the donor ewe had apparently died a few years ago, thereby precluding pertinent immunological testing of genetic relationships.

If we are to try to seriously analyze the mammalian cloning issue and its human implications, we should ask for details on points such as these, and for stronger statistics plus independent confirmation, before considering cloning of adult cells by means of nuclear transfer as a fait accompli.

Discussing such issues before they are immediately upon us is correct. However, indulging in endless debates is less so, when one considers both the scientific weaknesses of the experiment and the possible impact on the societal credibility of science itself of the "facts" on which they are purportedly based.

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Response: With reference to the skepticism of Sgaramella and Zinder about the origins of Dolly the sheep, we would like to provide clarification about some of the points they raise. Dolly is the only live birth that resulted from the transfer of nuclei from the adult-derived mammary cell cultures. Admittedly, a single birth from 400 attempted fusions is not an efficient system. However, the suggestion that "experimental error does occur" can be answered in a number of ways. First, Dolly is a Finn Dorset ewe. At the time of these experiments there were no other Finn Dorset cells being cultured in the laboratory and no Finn Dorset embryos being used in any other experimental system. Thus, in terms of breed, Dolly can only have been derived from the cell culture established from the mammary gland. These cell cultures were not established for the purposes of nuclear transfer, but had been previously isolated for other studies. The reason that a pregnant donor ewe was prepared was to establish, in culture, a cell line that exhibited mammary epithelial-specific characteristics for long-term culture. This was part of a collaboration between PPL Therapeutics and the Hannah Research Institute. For this reason, the genotypes of the fetus and the ram used for insemination were not analyzed, and no fetal material was retained for analysis.

Microsatellite analysis of Dolly mirrored exactly the pattern observed at both early (pre-nuclear donor) and late (post-nuclear donor) passages of the cell population. In addition, the cell population was predominately epithelial in nature. It is inconceivable that during the very short period of cell expansion, a rare fetal cell, if present, could have overgrown the mammary culture.

With regard to the mitochondrial DNA, samples from Dolly, all of the other lambs produced by nuclear transfer, the cell cultures, and representative samples from a number of randomly selected Blackface ewes (the breed used as oocyte donors) have been provided for analysis by independent third parties. When the results of these studies are available, they will be announced to the scientific community. Similarly, studies of the telomere length of the donor cells used for the production of Dolly, of Dolly herself, and of Finn Dorset sheep of representative ages are being analyzed at two centers. These studies are being coordinated with studies of all of the nuclear transfer offspring produced from embryo- and fetal-derived cell populations, of the cell populations themselves, and of the naturally produced offspring of those animals that have reached sexual maturity and have been bred.

We would like to point out that the methods described (1, 2) have been duplicated successfully by using cell populations derived from embryonic material (3). Other groups are attempting to repeat the technology using fetal and adult cell populations. It should be realized that only 11 months have elapsed since publication of our results (2); if one takes into account the time period required for gestation in sheep (5 months), one sees that it is unlikely that other authors would yet have had time to complete similar experiments and publish data.

Retrospectively, we and our co-authors realize that if the use of these cells for nuclear transfer had been anticipated, the skepticism of Sgaramella and Zinder could have been allayed by reference to an original donor tissue sample deposited with a respected neutral third party.

We were always aware that there would be some skepticism about our results and have been greatly encouraged by the positive reaction of the scientific community. We would like to think that this reflects the integrity with which we are accredited by our scientific peers. To us, as practicing scientists, this accolade is of paramount importance.

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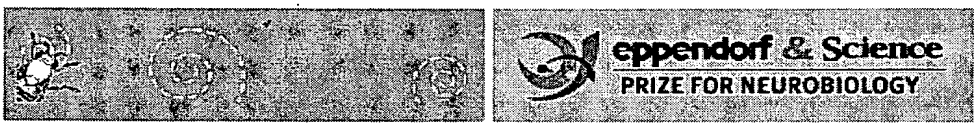

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